

Role of Human Desmoglein 3 in the Regulation of Cell Morphology and Motility via AP-1 and PKC Dependent Ezrin activation.

Submitted in partial fulfilment of the requirements of the Degree
of Doctor of Philosophy

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Collaborations, publications, awards and presentations

Collaborations

Mina Yang and Isa Cruz, UCL school of pharmacy, carried out mass spectrometry and analysis. Jarek Szary, Institute of Dentistry, designed and cloning truncated C-terminal Dsg3 construct. Ahmed Waseem, Institute of dentistry, provided the AP-1 luciferase constructs. Transwell invasion assay (Figure 4.6B) and Organotypic raft culture (Figure 4.7) was carried out by Emina Gunic, under the supervision of Dr Hong Wan. Organotypic culture analysis was carried out by Dr Hong Wan. Transwell migration assays (Figure 4.4) were carried out by Tania Mannan and Emina Gunic under the supervision of Dr Hong Wan. Luciferase assay using inhibitors (Figure 7.9) or Cos-1 and MDCK cells (Figure 7.8) cells carried out by Suzan Hama under the supervision of myself and Dr Hong Wan.

Manuscripts

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Dedicated to my father
Anthony Kirkwood Brown
(1960-2005)

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Abstract

Desmoglein 3 (Dsg3) belongs to the desmoglein subfamily and functions as an adhesion molecule in desmosomes. Two pools of Dsg3 have been identified, detergent soluble and insoluble proteins. Recent studies show that *DSG3* is upregulated in squamous cell carcinoma (SCC). However, its biological function in cancer remains poorly understood. The aim of this study was to investigate the extra-junctional functions of Dsg3, in particular its roles in signalling that regulates cell morphology and locomotion in cancer cells. This study adopted a unique cancer cell model with Dsg3 gain-of-function and has discovered two novel regulatory signal pathways that may play a crucial role in the control of cell invasion and metastasis in Dsg3 associated cancers. Firstly, Dsg3 regulates the phosphorylation of Ezrin at Thr567 in a PKC-dependent manner that is crucial for its activation and regulation of actin based membrane projections and accelerated cell locomotion in SCC. Secondly, Dsg3 modulates the transcriptional activity of cJun:AP1 that is responsible for regulating a cohort of genes to confer an invasive phenotype. It is likely that these two pathways are closely linked in that the Dsg3-mediated activation of cJun:AP1 elicits PKC-dependent Ezrin activation that in turn enable it to form a complex with Dsg3 at the plasma membrane to promote membrane projection and cell locomotion. Several lines of evidence support these conclusions: Dsg3 forms a complex with Ezrin at the plasma membrane and induces phosphorylation of Ezrin resulting in augmented membrane

protrusions and cell migration. Dsg3 silencing inhibits junction formation concomitant with collapse of membrane protrusion. Furthermore, Dsg3 regulates the activity of cJun:AP1. Collectively, these findings provide new insight regarding Dsg3 in cancer, suggesting it acts as a key regulator of cell invasion and metastasis in SCC. Therefore, targeting Dsg3 could be a potential new strategy in the control of cancer progression and metastasis.

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Abbreviations

AP-1	Activator Protein 1		
BIM	Bisindolylmaleimide I		
cAMP	Cyclic adenosine monophosphate	CRE	cAMP response element
CAR	Cell adhesion recognition		
Dp	Desmoplakin	Dsg	Desmoglein
Dsc	Desmocollin	DTD	Desmoglein terminal domain
EA	Extracellular anchor	EGTA	Ethylene glycol tetraacetic acid
ECM	Extracellular matrix	ERM	Ezrin radixin moesin
EDTA	Ethylenediaminetetraacetic acid	EMT	Epithelial to mesenchymal transition
EGFR	Epidermal growth factor receptor	Ez	Ezrin
FCS	Foetal calf serum	FRET	Fluorescence resonance energy transfer
GAPs	GTPases-activating proteins	(GDIs)	GTP/ GDP-dissociation inhibitors
GEF	Guanine nucleotide exchange factor	GSK	Glycogen synthase kinase
GEPs	GDP/GTP-exchange proteins		
IC	Intercellular C-terminal	IF	Intermediate filaments

	domain		
ICAM	Intercellular adhesion molecule	IMS	Industrial methylated spirit
IDP	Inner dense plaque	IPL	Intracellular proline rich linker domain
JNK	c-Jun N-terminal kinase		
KD	Knockdown	KDa	kilodalton
LEF	lymphoid enhancing factor		
MDCK	Madin darby canine kidney	MMP	Metalloproteinase
MET	Mesenchymal to epithelial transition		
NaCl	Sodium chloride		
ODP	Outer dense plaque		
PAGE	Polyacrylamide gel electrophoresis	PKC	Protein kinase C
PBS	Phosphate buffered saline	Pkp	Plakophilin
pERM	Phospho ERM	PLA	Proximity ligation assay
PF	Pemphigus foliaceus	PM	Plasma Membrane
Pg	Plakoglobin	PV	Pemphigus Vulgaris
RIPA	Radio-immunoprecipitation assay	RUD	Repeat unit domain
RT	Room temperature		
SCC	Squamous cell carcinoma	Ser	Serine
SD	Standard deviation	STAT	Signal Transducer and Activator of Transcription
SDS	Sodium dodecyl sulfate	SqCC	Squamous cell carcinom
TCF	T cell-specific transcription factor	Try	Tyrosine
Thr	Threonine	TTBS	Tris-buffered saline and tween 20
TRE	Tetracycline response element		

Chapter 1: Introduction

1.1 Metastatic cancer: the clinical perspective

In 2012 there were an estimated 32.6 million peoples living with cancer and 8.2 million cancer deaths worldwide (Globocan 2012 International Agency for research on cancer, WHO). The most common causes of cancer deaths being attributed to lung (1.59 million deaths), Liver (745 000 deaths), stomach (723 000 deaths), colorectal (694 000 deaths) breast (521 000 deaths) and oesophageal cancer (400 000 deaths) (Globocan 2012 International Agency for research on cancer, WHO). More than 90% of cancer related deaths are attributed to metastasis rather than the primary malignancy itself (Gupta and Massagué, 2006), underscoring the importance in tackling this aspect of cancer cell behaviour.

Desmoglein 3 has been shown to be upregulated in squamous cell carcinoma (SqCC) of the head and neck (HNSqCC) (Chen, et al., 2007, Chung, et al., 2004, Huang, et al., 2010), oesophagus (ESsqCC) (Fang, et al., 2014, Fang, et al., 2014, Savci-Heijink, et al., 2009) and lung (Savci-Heijink, et al., 2009), specifically non-small cell lung cancer (NSCLSqCC). Squamous cell carcinoma describe cancers of epithelial lineage occurring in organs covered with squamous epithelium such as the skin, mouth, oesophagus, prostate, lungs, prostate vagina and cervix. The majority of SqCC cases are attributed to the following anatomical sites (Yan, et al., 2011):

- Non-melanoma skin cancer (NMSCqCC),
- Head and neck (HNSqCC)
- Oesophageal (ESSqCC)
- Non-small lung cancer (NSCLSqCC).

Squamous cell carcinoma represent the most common cancer capable of metastasis worldwide (Marinkovich, 2007). The overall survival rate for these four SqCCs are among the lowest with a 5-year survival rate of <30%, 35% <10% and <14%, respectively, when systemic disease develops (Yan, et al., 2011) compared with the 4 major cancers in the UK (2010-2011) breast, prostate, lung and bowel with a 5-year survival of 87%, 85% 10% and 59% respectively (Cancer Research UK <http://www.cancerresearchuk.org/cancerinfo/cancerstats/survival/commoncancers/#One> July 2014). With the exception of NMSCC, which has it has the advantage of presenting on the skin surface and therefore greater chances of early detection, the overall survival among HNSCC, ESCC and NSCLC are among the lowest.

Treatment of SqCC has seen many developments. Targeted therapies most relevant to SqCC are being investigated which are aimed at cell cycle regulation, growth factor receptors, angiogenesis and protein degradation (Reviewed by (Yan, et al., 2011) which should, if proven successful, enhance current first line management strategies namely involving a combination of surgery, radiotherapy and chemotherapy. Despite advances in diagnostic and treatment methods, these four SqCC have not seen a significant increase in survival rates in 30 years (Agada, et al., 2009), this is in part believed to be attributed to late diagnosis and a lack of clinical and biological markers, which is essential to prognosis, monitoring tumour response to therapy, the development of resistance and metastasis. As a result most patients present with metastatic disease at the time of diagnosis and additional primary tumours which confers poor survival rates in the four major SqCC (Agada, et al., 2009, Yan, et al., 2011).

It is increasingly clear that the development of novel early detection strategies, prognostic markers and anti-metastatic therapies needs to be developed to increases the survival rate of patients that present with SqCC. In parallel, the understanding of

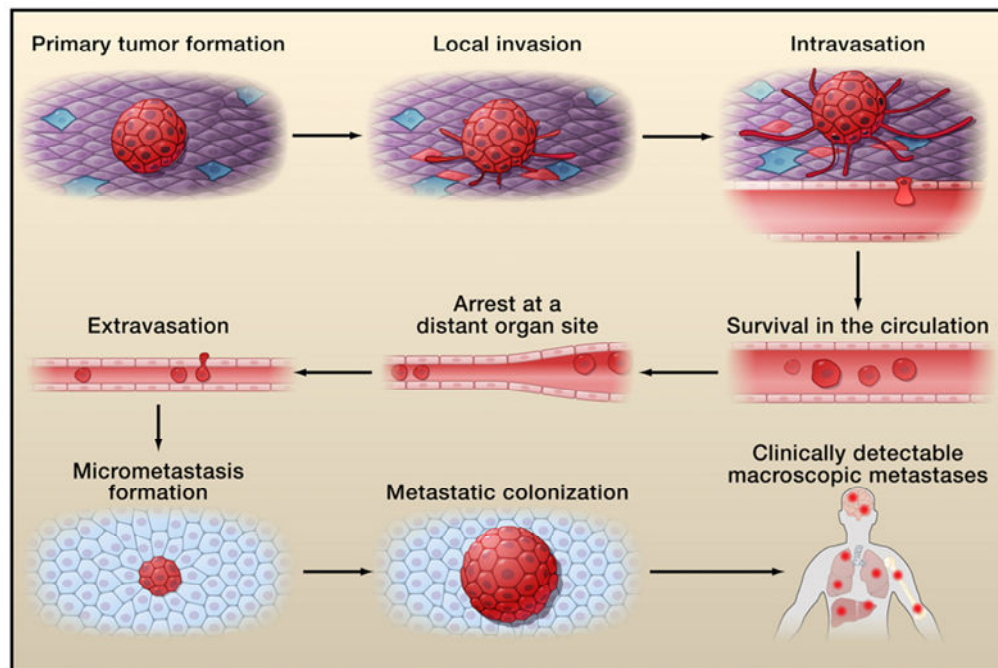


Figure 1.1 Invasion-metastasis cascade. Here tumour cells from the primary tumour site locally invade the surrounding tissues. Invasive tumour cells enter the blood stream or lymphatic system through intravasation. If the tumour cell survives in circulation they will arrest at a distant organ site and extravasate to enter the tissue. Tumour cells that survive at the secondary site form micrometastases which develop into clinically detectable macroscopic metastases. Figure adapted from (Valastyan and Weinberg, 2011)

identified targets/markers, beyond their efficacy and clinical impact, in cancer cell biology is essential to tackle therapeutic resistance and relapse. (For a details review of SqCC epidemiology, pathology, risk factors, molecular characteristics, prognostic markers and therapies see (Yan, et al., 2011)

1.2 Metastatic cancer: the cellular mechanisms

Invasion-metastasis cascade

Metastasis, by definition, describes the ability of a tumour cell to migrate and invade from the primary site. The process of metastasis is achieved through a complex series of cellular events termed the invasion-metastasis cascade. As summarised in Figure 1.1, the process requires a primary tumour cell to locally break through the basal membrane and invade the surrounding stroma or extracellular matrix (ECM). The cell then needs to intravasate into the circulatory or lymphatic system for transport, survive in circulation and arrest at a distal site. Once arrested the tumour cell extravasates into the parenchyma of the distal tissue to survive and adapt to the new microenvironment. Finally, the tumour cell initiates the proliferative programme at the metastatic site resulting in the formation of clinically detectable metastatic colonies or

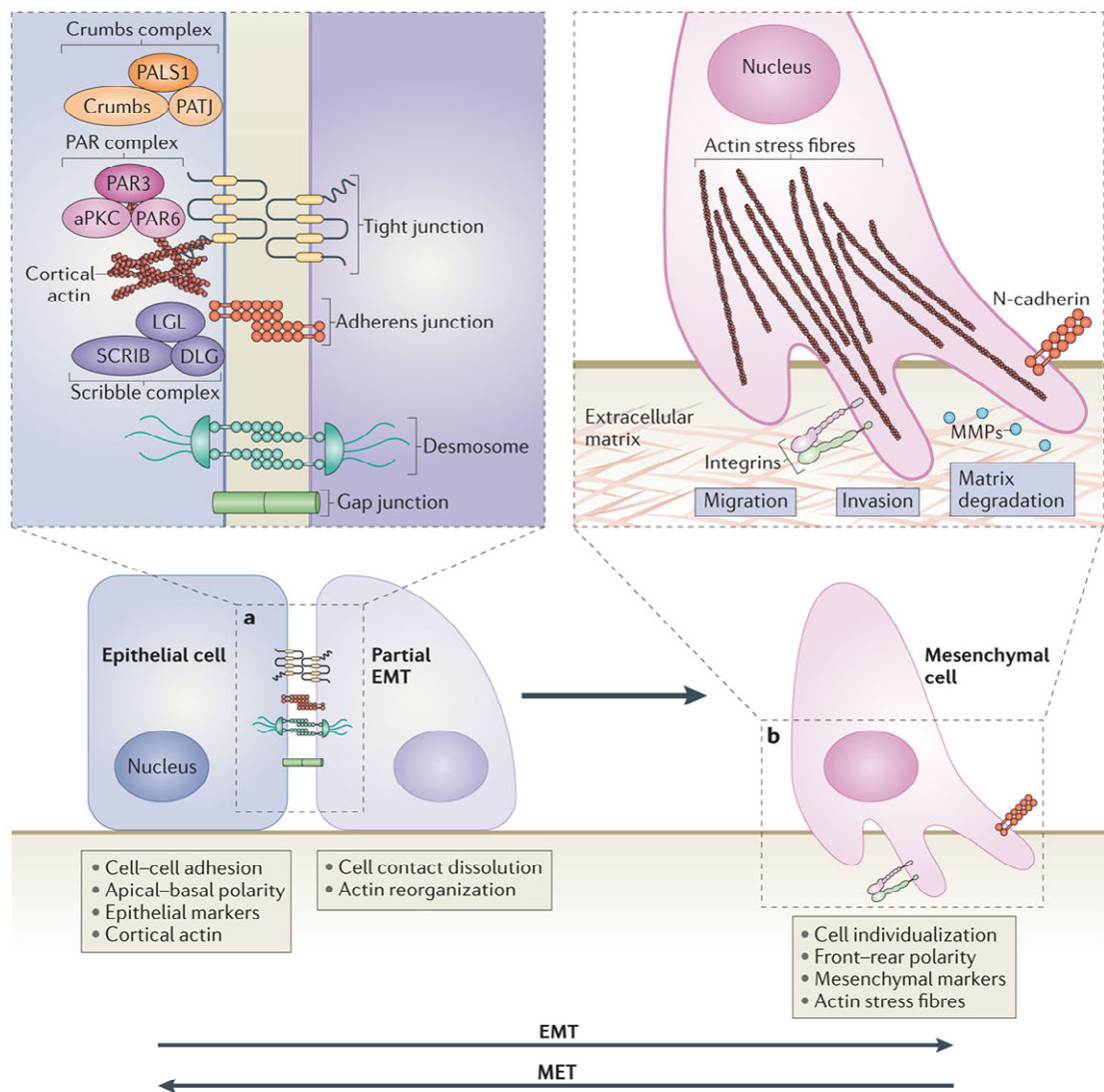


Figure 1.2 Cellular events of epithelial to mesenchymal transition. (A) Initiated by the loss of cell-cell adhesion through the disassembly of tight junctions, adherens junction, Desmosomes and gap junctions. Loss of cell-cell junctions in particular adherens and tight junctions disrupts polarity complex function resulting in a loss of apical basal polarity. Once EMT is initiated expression of junctional proteins and polarity complexes is repressed to maintain junctional and polarity loss concomitantly mesenchymal gene expression profiles are activated. **(B)** Progression of EMT sees actin re-organisation and the formation of pro-migratory and invasive membrane structures namely, lamellipodia, filopodia and invadopodia together with the expression of MMPs to degrade the ECM to facilitate motility and invasion. In the Reverse, mesenchymal to epithelial transition (MET) cells that have undergone EMT can revert back to an epithelial phenotype (Figure from (Lamouille, et al., 2014)).

macrometastases. (for a more detailed description of the cellular and molecular mechanism involved in the metastatic cascade see (Reymond, et al., 2013))

Epithelial to mesenchymal transition

The reversible process of epithelial to mesenchymal transition (EMT) describes the transition of polarised epithelial cells to assume a mesenchymal phenotype (Hay,

1995). As illustrated in Figure 1.2 this process can occur fully or partially involving cellular changes such as the loss of cell adhesion and apical-basal polarity; cytoskeleton reorganisation, changes in cell shape, development of pro migratory and invasive membrane structures; the acquisition of front-rear polarity and the expression of matrix degrading enzymes (Thiery, et al., 2009). Transcriptionally, epithelial gene expression signatures are down regulated and a mesenchymal gene profile is activated (Thiery, et al., 2009). All these events enable epithelial cell to assume a mesenchymal phenotype with enhanced migratory and invasive capacity, resistant to apoptosis and senescence (Thiery, et al., 2009). In the same way cells that have undergone this process can revert to an epithelial phenotype through the process of mesenchymal to epithelial transition (MET). The ability of cells to move back and forth between mesenchymal and epithelial states is essential during embryogenesis organ development and cancer progression specifically the establishment of secondary tumours, through the process of EMT and MET (Lee, et al., 2006). For more detail on the cellular and molecular events of EMT see (Kalluri and Weinberg, 2009, Lamouille, et al., 2014).

The process of EMT occurs in 3 distinct biological settings and thus classified into three different subtypes. Type 1 EMT occurs during embryo formation, organ development to generate diverse cell types. Type 2 is associated with wound healing, tissue regeneration and inflammation. When Type 2 EMT is sustained due to persistent inflammation, tissue fibrosis can occur. Finally Type 3 EMT occurs in association with cancer progression and metastasis. Here neoplastic cells i.e. those that have already accumulate genetic and epigenetic changes which confer a proliferative advantage to form a localised tumour, enter in to EMT to acquire characteristic essential to cancer progression and metastatic spread (Thiery, 2002). Typically these cells are located at the invasive front of primary tumours (Thiery, 2002) and would eventually enter into the invasion-metastasis cascade to disseminate to distal sites. Once arrested at a distal site cancer cells would enter into the process of MET to establish a secondary epithelial tumour (Zeisberg, et al., 2005) (For a more detailed description of the three EMT Subtypes see (Kalluri and Weinberg, 2009, Thiery, et al., 2009).

Epithelial to mesenchymal transition is regulated at transcriptional, translational and post-transcriptional levels. Key drivers of the EMT process are a variety of transcription

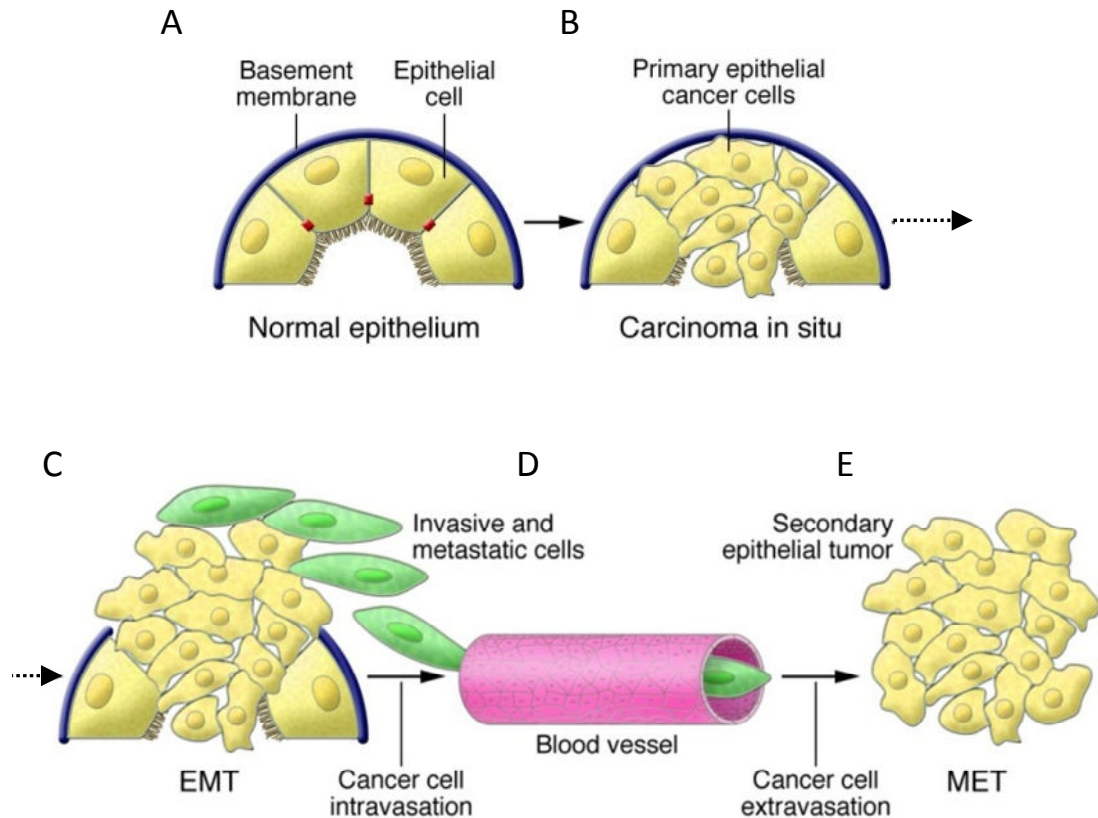


Figure 1.3 Role of EMT in cancer progression. As illustrated the EMT (C) occurs following the development of a carcinoma in situ (B) from normal epithelia (A). The EMT programme provides a mechanism for the acquisition of a malignant phenotype. The carcinoma cells that acquire a mesenchymal phenotype (green cell) which subsequently enter into steps on the migration-invasion cascade to leave the primary site, disseminate through circulation (D) to ultimately colonise distal organs following the process of MET (E) (adapted from (Kalluri and Weinberg, 2009)

factors including SNAIL, zinc-finger E-box-binding, AP-1 (see section 1.7) and basic helix-loo-helix (Lamouille, et al., 2014) which are intricately regulated in a cooperative fashion to repress the epithelial phenotype and activate the mesenchymal phenotype. (for a full review of the molecular mechanism and regulation of EMT see (Lamouille, et al., 2014).

1.3 Mechanism of tumour cell motility in metastasis

1.3.1 Modes cancer cell migration

To migrate and invade through surrounding tissue cancer cells must modify their shape and stiffness to interact with the surrounding microenvironment. How a cancer cells migrate and invade varies in both mode and strategy, disseminating as single cells or as a collective, in an amoeboid or mesenchymal fashion as illustrated in Figure 1.4. During individual cell migration cancer cells can adopt either a mesenchymal or amoeboid

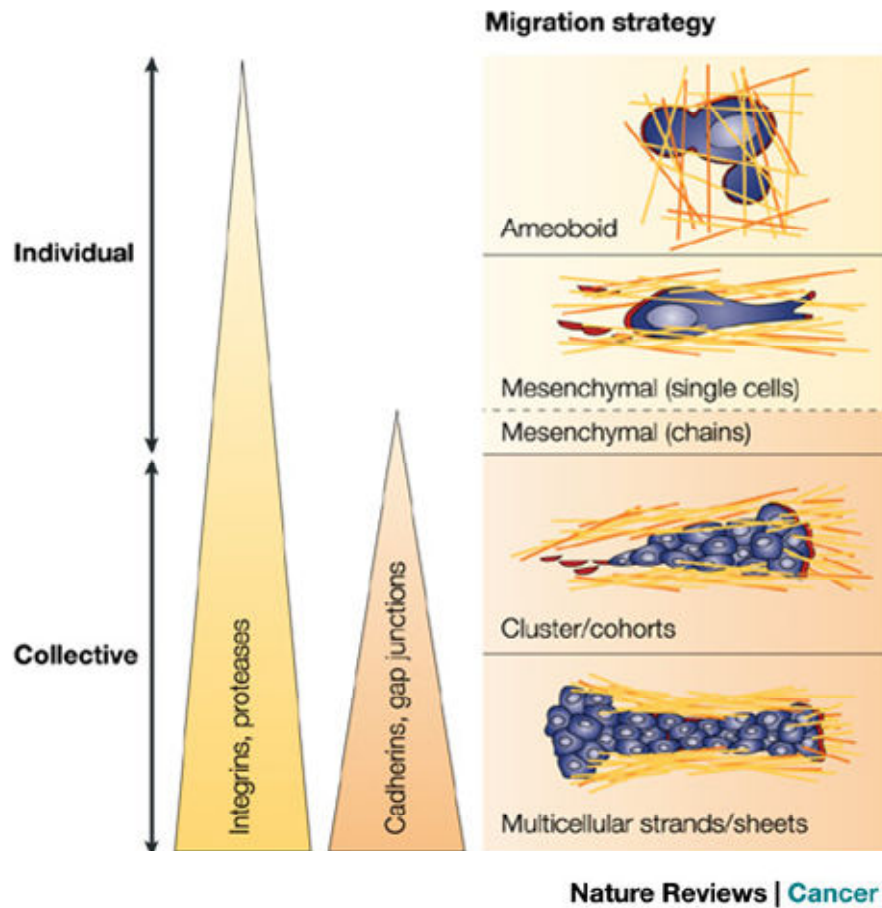
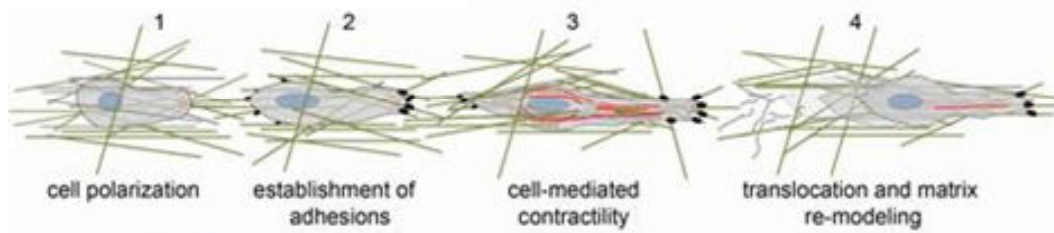


Figure 1.4 Strategies of cancer cell migration. This diagram illustrates the main migratory strategies adopted by cancer cell to facilitate cancer progression. Migration strategy is determined by the molecular programmes activated. As indicated by the triangles, collective migration is Cell-cell and Cell-ECM dependent while Individual cell migration is independent of intercellular adhesion. (Adapted from (Friedl and Wolf, 2003)

strategy. Amoeboid migration is characterised by cycles of morphological expansion and contraction, deformability and low substrate binding that is stress fibres- proteolysis- and integrin-independent (Friedl, et al., 2001). The force in this type of movement is generated by actomyosin which generates hypostatic pressure to essentially squeezing the cell body through interstitial gaps (Wolf, et al., 2003) (Figure 1.5). A less adhesive strategy, amoeboid migration is commonly adopted by lymphomas and small-cell lung cancer (Rintoul and Sethi, 2001).

Mesenchymal single cell migration requires a 5 step migration cycle predominantly found in cancers derived from connective tissue and epithelial origin (Brabletz, et al., 2001, Friedl and Wolf, 2009, Sanz-Moreno, et al., 2008). This strategy is dependent on the presence of focal contacts, actomyosin contractility and focalised proteolysis, thus integrin- and Rho GTPases- dependent (Friedl, et al., 1998, Sanz-Moreno, et al., 2008).

Mesenchymal migration



Amoeboid migration

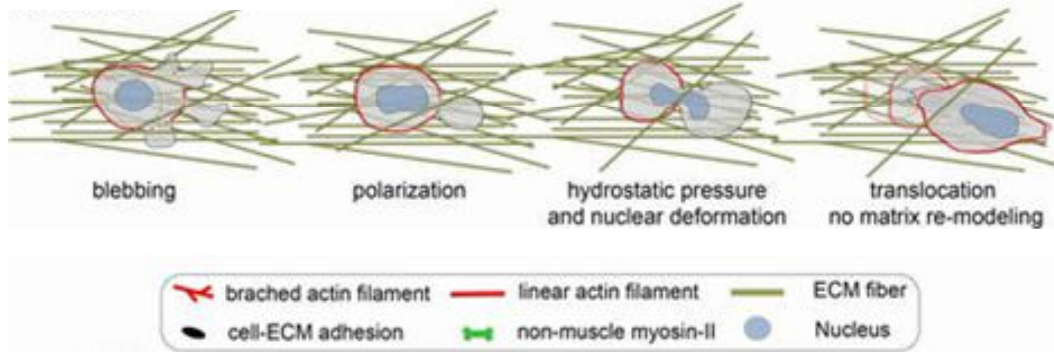


Figure 1.5 Major steps of mesenchymal and amoeboid migrations strategies. In mesenchymal cell migration the protrusions (lamellipodia, filopodia and pseudopodia) extend out into the ECM at the leading edge of a polarized cell. Focal contacts between the Cell and ECM form at the leading edge and rear of the cell. (1,2). Next Actomyosin contraction create tension force between the front a rear focal adhesions (3) Finally focal adhesion at the rear disassemble and the trailing edge retracted resulting in cell-body translocation forward. In Amoeboid migration polarised blebs form due to local disassembly of the cell actin cortex (1,2). Actomyosin contraction generates hydrostatic pressure squeezes the cell mass through interstitial gap (3). Only once the nucleus has cleared will the cell body fully translocate through the interstitial gap (Adapted from http://www.ltnt.ethz.ch/research/interface/cell_migration and (Friedl and Wolf, 2010)

As illustrated in Figure 1.5 mesenchymal migrations is achieved through the continuous cycle of interdependent steps. As shown the cell body changes shape to become polarized (front-rear) and elongated, forming extending membrane protrusions (pseudopodia) at the leading edge. Attachment of these protrusions to the surrounding ECM is then facilitated through focal adhesion. Once focal contacts at the leading edge and rear are established actomyosin contraction generates tension forces between the front and rear. Detachment of focal adhesions and retraction at the rear result in cell-body translocation forward (Friedl and Wolf, 2009). Rear focal contact then re-established and the cycle continues. The additional step of ECM proteolysis occurs in 3 dimensional rather than in two dimensions where cancer cell move over an organ surface for example. In a three dimension microenvironment focalised proteolysis at the leading edge is required to create a pathway for migration (Egeblad and Werb, 2002, Wolf and Friedl, 2011).

An additional mode of individual cell mesenchymal is as a chain (chain migration) which describes the stream-like movement of cells one after the other. The tracks through which these cellular chains or strands migrate are generated by the first invading cell or 'gureilla cell' clearing a path for subsequent cells to (Friedl and Wolf, 2008, Kulesa and Gammill, 2010). These chains of single cells are characteristic of epithelial carcinomas conferring high metastatic capacity and poor prognosis (Seftor, et al., 2002).

The alternative mode of cancer cell migration and invasion is collective, here two morphological and functional variants have been described, the cluster/cohort strategy and the multi-cellular strand/sheet strategy (Figure 1.4). Cluster/cohort migration describes the migration of detached cell clusters from the primary site, which characteristically move through tissue gaps i.e. the path of least resistance seen in epithelial cancers. The multi-cellular strand/sheet strategy describes the protrusion of sheets and strands of tumour cells which maintain contact with the primary site seen in squamous cell carcinoma, mammary and colon cancers (Ilina and Friedl, 2009, Nabeshima, et al., 1999).

The mode and strategy adopted by any cancer cell is dependent on the molecular programme utilised and surrounding microenviroment (Friedl, 2004, Friedl and Wolf, 2010). Both of which are subject to change due to cancer cells migrating and invading into different microenvironments and ECM compositions; and changes in the cancer cell itself through processes including EMT, MET and the invasion-metastasis cascade which dramatically alter cell morphology, junction protein expression and proteolytic capacity all of which will provoke adaptive re-programming to engage in a migration strategy best suited to the new context (Hanahan and Weinberg, 2011, Polyak, et al., 2009, Wolf and Friedl, 2011). This migrational plasticity means that cancer cells are able to shift from highly adhesive, proteolytic migration strategies (mesenchymal) to low adhesive, non-proteolytic migration (amoeboid); and from collective to detached and disseminated migration (EMT). For more detail on modes and strategies of cancer cell migration and invasion see (Friedl and Alexander, 2011, Friedl and Wolf, 2003, Sahai, 2005).

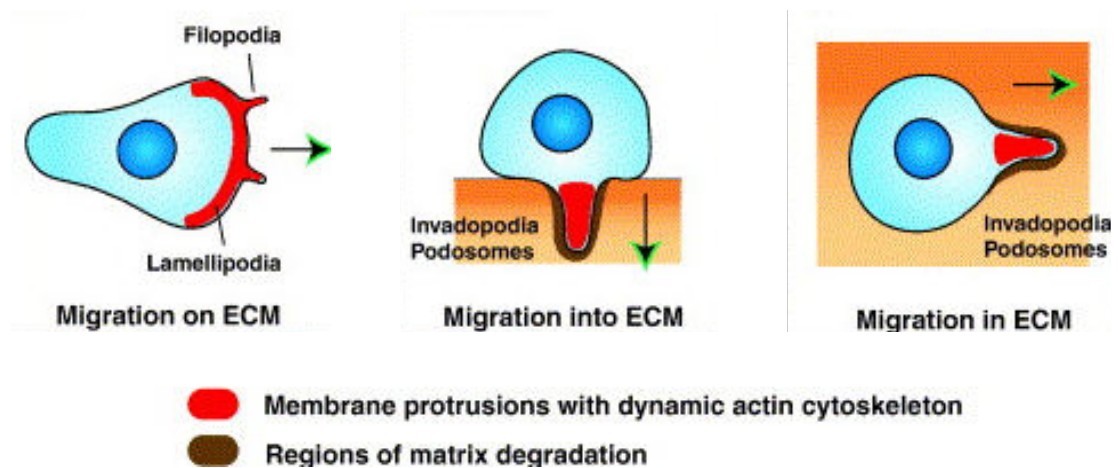
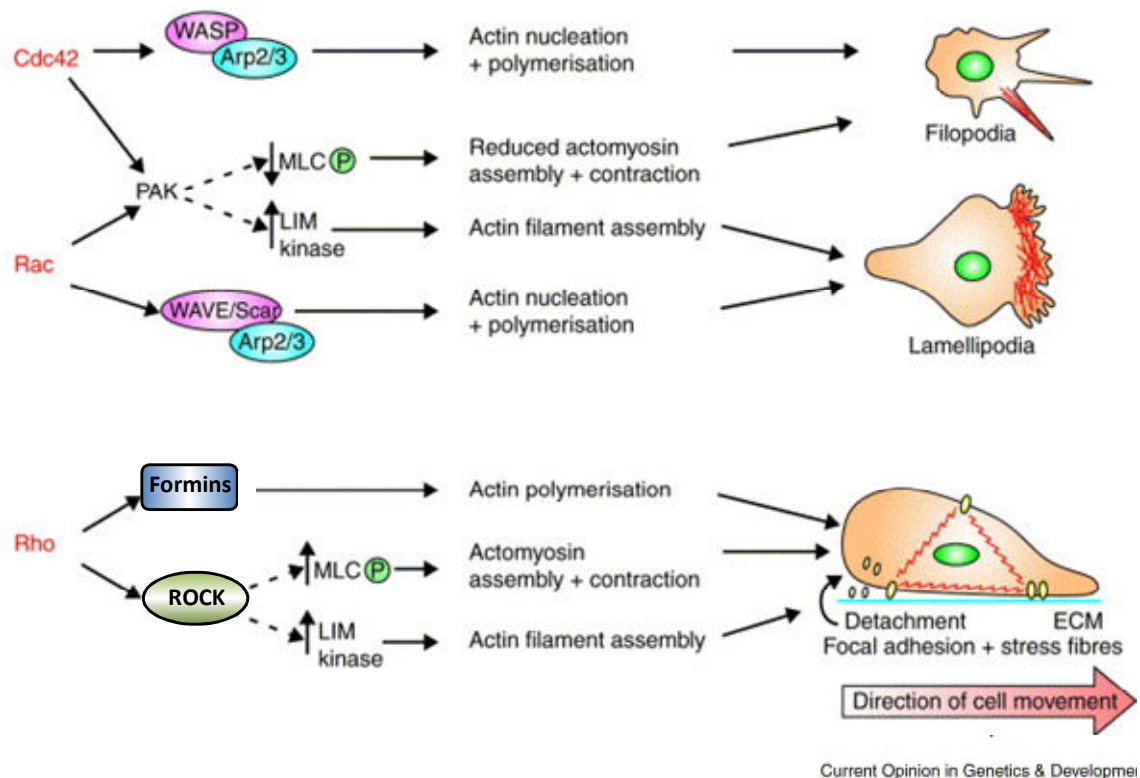


Figure 1.6 Actin based membrane protrusions. There are 3 main types of actin based processes driven by localised actin polymerisation, exhibited by migrating and invading cells Lamellipodia, Filopodia and Invadopodia/Podosomes. Lamellipodia and filopodia are predominantly utilised by cell migrating on two dimensional substrates to facilitate motion. However on three dimensional ECM, migration requires the cell to degrade and remodel the surrounding ECM in order to progress, hence these cells require the formation of invadopodia eliciting their proteolytic activity to invade and migrate (Figure from (Yamaguchi and Condeelis, 2007)).

1.3.2 Molecular mechanisms and signalling pathways in cancer cell migration

Actin cytoskeleton and actin based structures

Cell migration is a highly integrated multistep process initiated by actin based processes, specifically membrane protrusions (Bailly and Condeelis, 2002) with the aberrant regulation of migration promoting disease and plays a pivotal role in cancer progression and metastasis (Sahai, 2005). The driving force behind the formation and maintenance of pro-invasive and -migratory membrane structures is the spatial and temporal regulation of the actin cytoskeleton, specifically, actin filament assembly, disassembly and contraction at specific sub-cellular domains such as the leading edge and rear of a polarised cells (Pollard and Borisy, 2003, Sahai, 2005). The protrusive membrane structures, lamellipodia, pseudopodia, filopodia and invadopodia, formed by migrating and invading cells are classified by their morphological, structural and functional characteristics (Figure 1.6). Lamellipodia are flat sheet like projections which form at the leading edge of migrating cells. Characterised by a dense network of branched actin filaments lamellipodia formation is driven by Scar/WAVE and Arp2/3 complex activation (Pollard and Borisy, 2003, Svitkina and Borisy, 1999). They have a major role in driving cell migration as they extend and attached to the



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Figure 1.7 Regulation of cell migration by Rho GTPases. Illustrated in a schematic demonstrating the Role of Rho GTPases in regulation of cell motility through their ability to modulation, filopodia, lamellipodia, actomyosin contraction and stress fibres. Cdc42 promotes filopodia formation through the activation of WASP/N-WASP proteins and Arp2/3 complex activation. Rac proteins promote lamellipodia formation through WAVE/Scar proteins and Arp2/3 complex activation. Together Cdc42 and Rac spacially down regulate actomyosin assemble and contractility at the leading edge via the PAK/MLC (myosin light chain), while promoting actin polymerisation through PAK/LIM kinase. Rho protein promote cell-body translocation through modulating Actomyosin assembly, stabilisation and contractility through LIM kinase, formin protein activation, ROCK-dependent MLC phosphorylation and respectively (Adapted from (Frame and Brunton, 2002, Keely, et al., 1998)

surrounding ECM via focal adhesion providing an anchor from which force can be generated through actomyosin contraction to pull the cell body forward (Condeelis and Segall, 2003). Pseudopodia are the functionally and compositionally equivalent to lamellipodia differing only in structure and context to form cylindrical, rounded or filopod-like protrusions in three-dimensional matrix, whereas lamellipodia are associated with two dimensional migration (Sahai, 2005). Filopodia are transient thin finger-like projections that contain parallel bundles of crosslinked actin filaments which also form at the leading edge of migrating cells (Faix and Rottner, 2006). Their formation is driven by members of the formin family via WASP/N-WASP AND Arp2/3 complex activation (Pellegrin and Mellor, 2005, Schirenbeck, et al., 2005). Filopodia are thought to sense external cues to determine migration direction rather than facilitating migration itself (Gupton and Gertler, 2007). Invadopodia/Podosomes are

ventral membrane protrusions characterised by the presence of matrix degradation enzymes (Buccione, et al., 2004). These protrusions facilitate migration through dense ECM through the degradation and remodelling of ECM structures (Even-Ram and Yamada, 2005), thus formed by highly invasive cancer cells in three dimensional environments (Buccione, et al., 2004). Invadopodia formation is driven by N-WASP-Arp2/3 and cofilin pathway activation (Yamaguchi, et al., 2005).

Finally, actin cytoskeleton contractility is an essential element in the process of cell migration as the ability of cells to move requires the generation of force. This is achieved through the association of F-actin with the actin motor protein myosin II to form actomyosin. The process of contraction occurs through binding of ATP and its subsequent hydrolysis for Myosin II of actomyosin to generate contraction forces between the front and the rear of the cell to facilitate cell-body translocation in the direction of movement (Cramer, 1999, Kamm and Stull, 2001).

Rho GTPases

The driving force behind all actin-based strategies of migration and invasion is the actin cytoskeleton through its co-ordinated spatial and temporal reorganisation, assembly, disassembly and contractility (Pollard and Borisy, 2003). The Rho GTPase family consists of 20 small GTP-binding proteins of which Rho, Rac and Cdc42 subfamilies represent the most widely studied in cell migration (Noren, et al., 2001). Rho, Rac and Cdc42 family proteins have been shown to play an essential and pivotal role in regulation of pathways leading to changes in cell shape, formation of stress fibres, focal adhesion, membrane protrusions and cell migration as a whole (Raftopoulou and Hall, 2004, Sahai and Marshall, 2002). Rho GTPases exist in two interchangeable forms. GTP bound (active) and GDP bound (inactive). GDP/GTP-exchange proteins (GEPs) stimulate the exchange of GDP to GTP/GDP-dissociation inhibitors (GDIs) inhibit the exchange of GDP to GTP; and GTPases-activating proteins (GAPs) stimulate the exchange of GTP to GDP (Braga and Yap, 2005, Menke and Giehl, 2012). In their GTP bound state Rho GTPase interact with specific downstream effectors to provoke a cellular response.

As illustrated in Figure 1.7 Rho GTPases play an essential role in cell migration through the regulation of filopodia and lamellipodia formation; focal adhesion and actomyosin

contraction. As shown both Cdc42 and Rac are required at the leading edge of migrating cells promoting the formation of filopodia and lamellipodia respectively. This is achieved through the WASP/Scar/WAVE family proteins acting on Arp2/3 complex (Machesky and Hall, 1997, Nobes and Hall, 1995). The Arp2/3 complex nucleates actin polymerisation at sites of filopodia and lamellipodia formation (Machesky and Insall, 1998, Mullins, et al., 1998). Additionally Cdc42 and Rac down modulate actomyosin assembly and contraction at the leading edge (Sanders, et al., 1999) to facilitate its extension into the ECM to essentially co-ordinate cellular effort towards a net forward movement. These inhibitory effects are achieved through p21-activating kinase (PAK) activation (Daniels and Bokoch, 1999). PAK is a serine threonine kinase which phosphorylates and inactivates myosin light chain kinase (MLCK) to reduce actomyosin contraction at the leading edge. MLCK activates Myosin II, in its active state myosin II binds F-actin to generate actomyosin contraction (Verkhovsky, et al., 1995). Similarly PAK can also phosphorylate and inactivate LIM kinase which phosphorylates and inactivates de-polymerisation protein cofilin to promote actin filament assembly (Frame and Brunton, 2002). Rho protein function in cell migration is primarily associated with actomyosin contractility, essential to cell body contraction and rear retraction (Burrage and Chrzanowska-Wodnicka, 1996, Chrzanowska-Wodnicka and Burrage, 1996). This is achieved through its ability to, via ROCK, activate LIM kinase (Maekawa, et al., 1999, Sumi, et al., 2001); inactivate LIM phosphatases (Kawano, et al., 1999) which stabilises actin filaments and actomyosin bundles to collectively promote the generation of contractile forces. Rho proteins also activate formin proteins such as mDia1 and mDia2 which promote actin polymerisation through their association with actin monomer binding protein, profilin (Gotoda, et al., 2000, Uehata, et al., 1997). (For comprehensive reviews on the role and regulation of Rho GTPases in cell migration and invasion see (Frame and Brunton, 2002, Raftopoulou and Hall, 2004, Sahai, 2005, Sahai and Marshall, 2002))

1.4 The role of Intercellular junctions in tumour suppression

Intercellular junctions mediate adhesion between adjacent cells. There are several types of intercellular junctions, anchoring, communicating and occluding junctions (Figure 1.8). Intercellular junctions are particularly abundant in the epidermis and confer characteristics critical to its function. Desmosomes and adherens junctions are

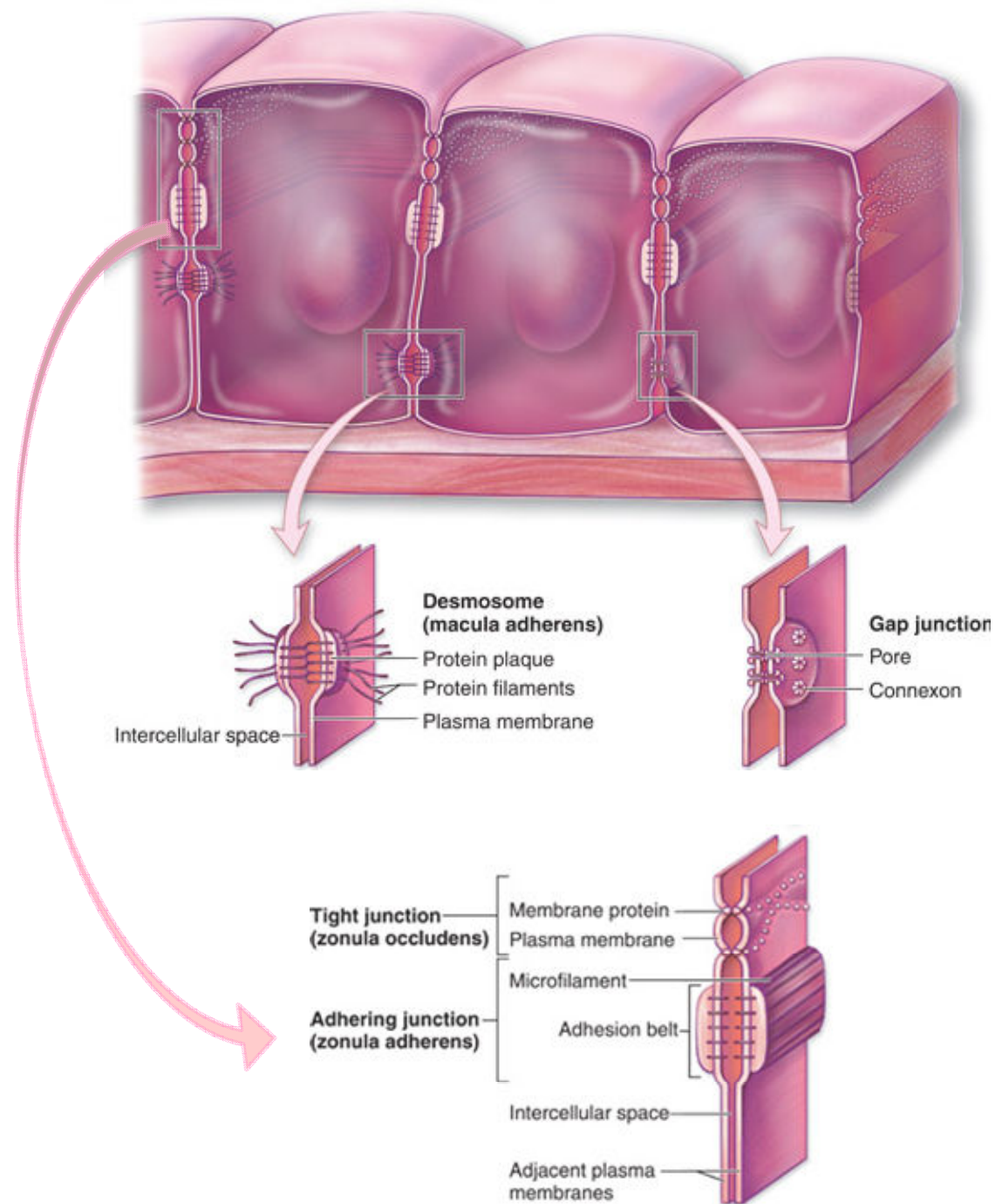


Figure 1.8 Cell-Cell Junctions. Tight junctions are occluding junctions located at the apical membrane providing a selectively impermeable barrier to seal the intercellular space. Tight junctions also play an essential role in the establishment of cell polarity through their association with polarity complexes. Adherens junctions located more basal than tight junctions they junction to link the actin cytoskeleton of neighbouring cells to sites of adhesion forming an adhesion belt which encircles the host cell or form spots of adhesion. Desmosomes form spots of adhesion linking the intermediate filaments of neighbouring cell to sites of adhesion which is essential to tissue architecture and integrity. Finally Gap junctions, form spots of adhesion, with gabs between them spanning ~2nm bridged by ion channels composed of connexons to directly link the cytoplasm of neighbouring cells. (Adapted from <http://organelle-marker.com/intercellular-junctions/>. August 2014)

both anchoring junctions and confer mechanical strength and integrity essential to the maintenance of tissue architecture. Gap junctions allow cell-cell communication by facilitating the movement of molecules and ion between adjacent cells, crucial to coordinated differentiation and proliferation. Finally occluding Tight junctions, provide a selectively impermeable barrier to solutes and water between epithelial cells and provide essential barrier functions.

Intercellular junctions are conventionally accepted to function as tumour suppressors by restricting cell growth through cell-adhesion-mediated contact inhibition (Okegawa, et al., 2002). In support the disruption of cell-cell adhesion has been shown to significantly contribute to uncontrolled cell proliferation and play a causal role in tumour dissemination. The tumour suppressor function of adhesion is exemplified by the function of E-cadherin which when disrupted is associated with tumour development (Pećina-Slaus, 2003). When expressed in cancer cell lines, E-cadherin inhibits growth and reduces invasiveness (Frixen, et al., 1991, Vleminckx, et al., 1991, Watabe, et al., 1994). Likewise the expression of desmosomal components in non-adhesive fibroblasts (L929) generates adhesion, while the strongly invasive nature of these cells was inhibited when transfected with desmosomal components. Furthermore, the invasive phenotype of L929 fibroblasts was restored when desmosomal adhesion was inhibited (Tselepis, et al., 1998). Recent studies support this concept by identifying mutations in cell-cell adhesion genes encoding alpha-catenins (component of adheren junctions) in Head and Neck SqCC (Fanjul-Fernández, et al., 2013). Further prognostic assessment to evaluate the impact of these alpha catenin mutations revealed four-fold increase in mortality if tumours harbour these lesions (Fanjul-Fernández, et al., 2013). Another study shows further support by demonstrating that loss of cell adhesion molecule, Echinoid, promotes tissue over growth i.e. loss of contact inhibition function (Yue, et al., 2012). Specific to Desmosomes, the loss or reduction of junction components including Dsg1-3, Pg, and Pkps has been observed in the development and/or progression of SqCCs of the skin head and neck and prostate, correlating with increased metastasis and poor prognosis. (Dusek and Attardi, 2011). For more detail on the role of cell adhesion in tumour suppression see (Dusek and Attardi, 2011, Moh and Shen, 2009).

1.5 Desmosomes

Desmosomes are calcium dependent, highly organised multi-protein structures composed of cadherins, armadillo and plakin family proteins. They uniquely function as intercellular adhesive junctions which tether the intermediate filaments of neighbouring cells to the plasma membrane at sites of cell-cell contact to form the Desmosome-Intermediate Filament Complex (DIFC) (Delva, et al., 2009, Getsios, et al., 2004, Saito, et al., 2012). Desmosomes provide mechanical and adhesive strength which is essential to the maintenance of tissue architecture and integrity, particularly to that of the skin, oral mucosa, simple epithelia and heart (Desai, et al., 2009, Green and Simpson, 2007) which are subjected to relatively high levels of mechanical stress.

1.5.1 Desmosome structure and composition.

As illustrated in Figure 1.9, a characteristic desmosome is composed of: transmembrane cadherins, desmogleins (Dsg) and desmocollins (Dsc), which mediate adhesion between adjacent cells; Aramdillo family proteins, plakoglobin (Pg) and plakophilin (Pkp), which associate with the cytoplasmic tail of cadherins; and plankin family protein, Desmoplakin (Dp), which associates with the stress-bearing intermediate filaments of the cytoskeleton. These components are arranged to form two symmetrical electron dense cytoplasmic plaques (DCP) from neighbouring cells, around a shared central extracellular core domain (ECD) which is bisected by a dense midline (Farquhar and Palade, 1963, Odland, 1958). The extracellular core domain (also known as desmoglea) spans 20-35nm and consists of the extracellular domains of desmosomal cadherins. Each DCP consists of an inner dense plaque (IDP) and an outer dense plaque (ODP) both approximately 15-20nm thick. The cytoplasmic tails of the desmosomal cadherins directly interact with Pg, also known as γ -catenin, which in turn associates with the N-terminal globular head of Dp and Pkps to form the ODP. The IDP consists of the C-terminal tail of Dp which interacts with the intermediate filaments of the cellular cytoskeleton. Collectively they form the DIFC complex (Garrod and Chidgey, 2008).

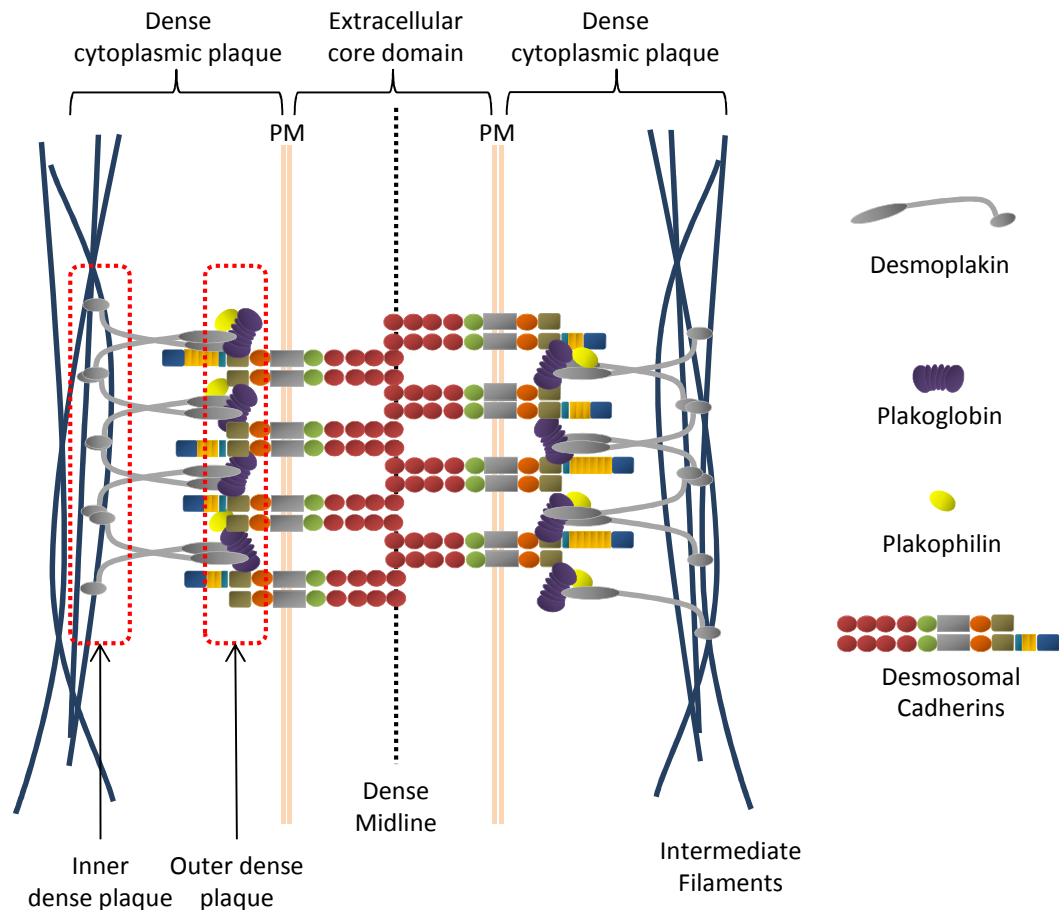


Figure 1.9 Desmosome structure. The desmosome is a multi-protein adhesive complex composed of desmosomal cadherins, armadillo family proteins (plakoglobin and plakophilin) and plakin family proteins (Desmoplakin). They are arranged as two symmetrical electron dense plaques (inner dense plaque and outer dense plaque) which facilitate the association between the C-terminus of the desmosomal cadherin and the intermediate filaments of the cytoskeleton. Desmosomes function to link the intermediate filaments of neighbouring cells to sites of adhesion which is essential in the maintenance of tissue integrity.

Desmosomal Cadherins

Desmosomal cadherins are calcium binding, single pass transmembrane glycoproteins that belong to the cadherin superfamily. Their function is to provide the adhesive Dsc isoforms can exist in 2 spliced variants, a 'long' (a) and 'short' (b) form, the latter of which lacks the ICS domain. The extracellular domain of desmosomal cadherins consists of four extracellular cadherins like domains (EC1-4), separated by calcium binding motifs, and an extracellular anchor (EA) domain followed by the transmembrane domain (TM). The intracellular domain consists of an intracellular anchor domain (IA), an intracellular cadherin-like domain (ICS) which provides a interface at the core of desmosomes. There are currently seven characterised human isoforms, Dsg 1-4 and Dsc 1-3, encoded by separate genes. As illustrated in Figure 1.10,

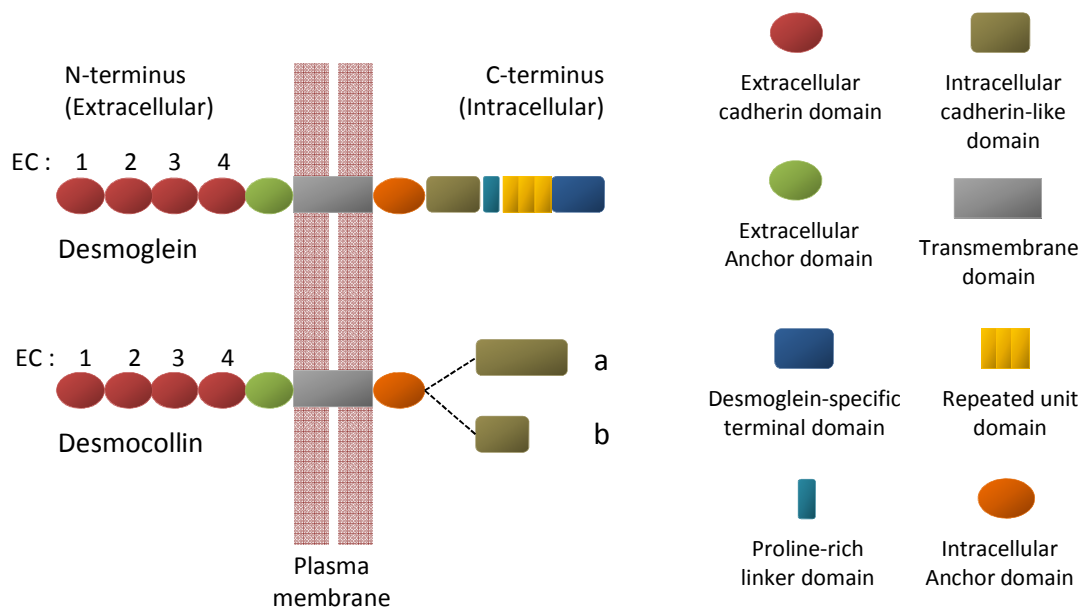


Figure 1.10 Desmoglein and Desmocollin proteins structure. Both Dscs and Dsgs share a common extracellular structure consisting of four extracellular cadherin like domains (EC), followed by an extracellular anchor domain (EA). The transmembrane domain (TM) links the extracellular domain to the intracellular domain that contains an intracellular anchor domain (IA) followed by a intercellular cadherins-like domain (ICS). The ICS is shortened in Dsc (b) variant. Specific for Dsgs are the additional three domains, a proline-rich linker domain (IPL), repeating unit domain (RUD) and finally a desmoglein-specific terminal domain (DTD) that are located at the C-terminus of Dsgs.

binding site for Pg, a proline-rich linker domain (IPL), repeating unit domain (RUD) and finally, a desmoglein-specific terminal domain (DTD) that is found only in Dsg proteins. The functions of IPL and RUD are currently unknown. For Desmosomal cadherins mediate adhesion through their Cell Adhesion Recognition (CAR) sites located in the EC1 domain (Tselepis, et al., 1998). Adhesion between the desmosomal cadherins was originally thought to occur in a homophilic manner like classical cadherins. However, later studies using blocking peptides corresponding to the CAR sites of both Dsgs and Dscs have shown that desmosomal adhesion was only perturbed when peptides to both Dsgs and Dscs were present (Runswick, et al., 2001), suggesting that desmosomal cadherins mediate heterophilic binding. A later report by the same group has shown that Dsc2/3 and Dsg2/3 also interact with each other in a homophilic and isoform specific fashion suggesting that both heterophilic and homophilic ligations can occur (Thomason, et al., 2010). For more detail on the structure of Desmosomal cadherins see (Al-Amoudi, et al., 2011, Kowalczyk and Green, 2013, Saito, et al., 2012).

Armadillo Family proteins

Armadillo proteins form a large family characterised by the presence of a repeated ~42 amino acid ARM motif, such proteins include β -catenin, Pg and Pkps. Plakoglobin, consists of an N- and C-terminal domain which flank a central right handed superhelix (Huber, et al., 1997) that associates and binds to the C-terminal tails of desmosomal cadherins. Plakoglobin is a homologue of β -catenin, exhibiting dual localisation in desmosomes and adherens junction through its association with desmosomal cadherins and E-cadherin. However, due to higher affinity, Pg tends to localise in the desmosome (Chitaev, et al., 1996). Plakoglobin has also been implicated in facilitating cross-talk between desmosomes and adherens junctions and in regulating desmosome size (Lewis, et al., 1997, Palka and Green, 1997, Ruiz, et al., 1996).

Plakophilin exists as 3 isoforms (Pkp 1-3) expressed in a tissue- and cell differentiation-specific manner (Hatzfeld, 2007). Plakophilin 1 and 2 have two variants, 'a' the shorter variant and 'b' the longer form. Structurally, Pkps consist of a central *arm* repeat domain with a flexible insert (Choi and Weis, 2005) flanked by C- and N-terminal domains. All Pkps exhibit dual localisations in desmosomes and the nucleus (Hatzfeld, 2007), regulated by 14-3-3 protein which retains cytoplasmic localisation (Müller, et al., 2003, Schmidt, et al., 1997). The N-terminal head of Pkp mediates interactions with Dp, Pg and desmosomal cadherins as well as facilitating their transport to cell-cell contacts (Chen, et al., 2002, Hatzfeld, et al., 2000, Kowalczyk, et al., 1999).

Desmoplakin

Desmoplakin is the predominant plaque protein required for desmosome assembly, intermediate filament association and regulation of desmosomes and adherens junction localisation (Bornslaeger, et al., 1996, Bornslaeger, et al., 2001, Huber, 2003, Smith and Fuchs, 1998, Troyanovsky, et al., 1994). Desmoplakin consists of a globular head and tail domain which flank a central α -helical coiled-coil rod domain that mediates Dp dimerisation (Green, et al., 1988). The globular N-terminal head domain facilitates targeting to ODP and binding to Pg, while the C-terminal domain mediates attachment to intermediate filament (Bornslaeger, et al., 1996, Meng, et al., 1997, Stappenbeck and Green, 1992). There are two isoforms of Dp, DpI and DpII generated by alternative splicing, however they appear to be functionally redundant (McGrath, et al., 1997, Uzumcu, et al., 2006).

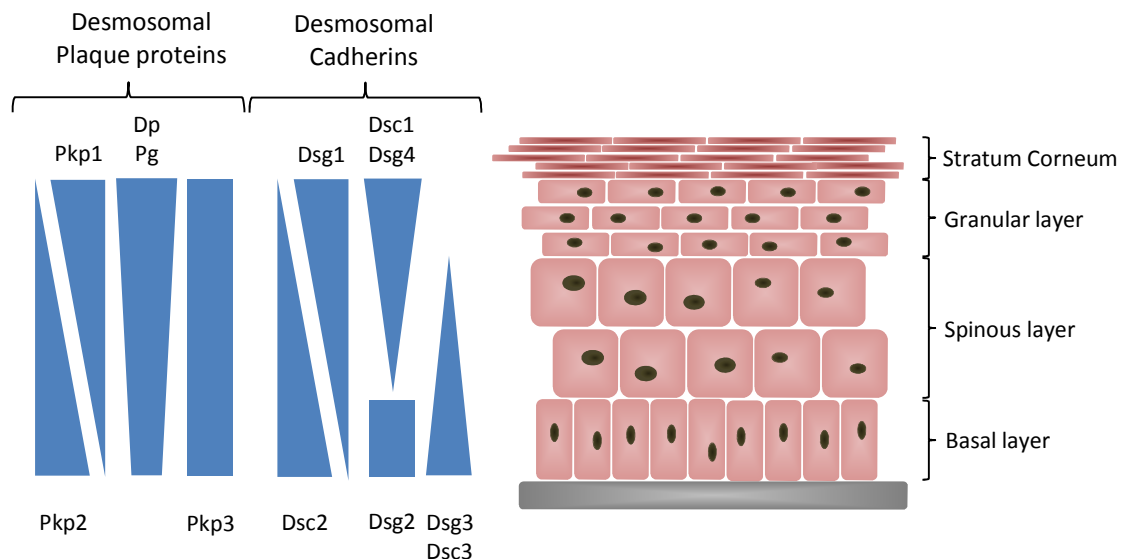


Figure 1.11. Expression of desmosome components in the epidermis. A schematic of the Basal, spinous, granular and cornified layers of the epidermis (right). The blue blocks on the left, illustrate the expression pattern of desmosomal cadherins (Dsg 1-4 and Dsc 1-3) and plaque proteins (Dp, Pg, and Pkp 1-4). The controlled levels of expression and localisation of the desmosomal components alters the composition of desmosomes formed, which is thought to be attributed to tissue differential and morphogenesis. (Figure adapted from (Getsios, et al., 2004, Green and Simpson, 2007))

Other desmosomal components

Perp is an essential component of desmosomes of stratified epithelia and heart (Ihrie, et al., 2005, Marques, et al., 2006) and is required for normal desmosome assembly and function in these tissues (Ihrie, et al., 2005). However, the precise role of Perp is not fully understood. Corneodesmosin is a secreted glycoprotein incorporated into desmosomes of the cornified layers of the epidermis during the process of epithelial cornification. Corneodesmosin is thought to act as an adhesion molecule in the cornified layers of stratified epithelia (Jonca, et al., 2002).

Desmosome composition varies with cell type and differentiation status. Such variation results in structural changes that are visible in desmosome appearance and size. Such tailoring is proposed to suit the specialised functions of the cells and tissues that possess them. This is well exemplified through the expression patterns of desmosomal components restricted to specific layers in the epidermis. As illustrated in Figure 1.11 Dsg1, Pkp1 are highly expressed in the differentiated cells of granular and spinous layers whereas Dsg3, Dcs3 and Pkp2 are predominately expressed in the proliferating basal and suprabasal layers. Dsg4 and Dsg2 exhibit more restricted expression in the

granular and basal layers, respectively, while Pkp3, Pg and Dp exhibit more ubiquitous expression through the epidermis.

1.5.2 Desmosome assemble, adhesion and regulation

Desmosome assembly

Initiated by cell-cell contact and adherens junction formation, desmosomes assemble to confer and stabilise strong intracellular adhesion (Lewis, et al., 1997). The precise sequence of events of desmosome assembly has been hindered by the relative insolubility of the junctions. However, technological advances including live-cell imaging and fluorescent reporters have begun to overcome this obstacle. As currently understood and illustrated in Figure 1.12, desmosome assembly occurs in several phases (Nekrasova and Green, 2013). Firstly, the desmosomal cadherins assemble at the plasma membrane as illustrated in Figure 1.8 A. Specifically, Dsc-enriched vesicles, which initiate assembly (Burdett and Sullivan, 2002, Collins, et al., 1995), followed by Dsg-enriched vesicles are transported to the plasma membrane associated with Dp in a microtubule-dependent fashion spatially and temporally coordinated by kinesin-2 and Pkp2 for Dscs and kinesin-1 for Dsgs (Green, et al., 2010, Nekrasova, et al., 2011). To complete assembly, targeting and tethering of these vesicles into the plasma membrane has been proposed to be facilitated through the Sec3 exocyst protein complex (Andersen and Yeaman, 2010)

The next phase is cytoplasmic plaque assembly. As summarised in Figure 1.8B this phase is already initiated during Dsg recruitment to the plasma membrane, where Dp in concert with Dsg3 accumulates at the cell cortex. This is quickly followed by the recruitment of non-membrane-bound Dp-containing particles associated with the IF cytoskeleton. Once at the cell cortex Dp is translocated to cell-cell contacts where it binds to the C-terminus of desmosomal cadherin via Pg in the formation of the IDP in a microtubule- and actin -dependent manner (Godsel, et al., 2005). The translocation of Dp-associated complexes is regulated and promoted by Pkp2 which acts as a scaffold for a complex containing plakins and PKC α which modulate the interaction between Dp and intermediate filaments. The contribution of Pg and other Pkps to the desmosome assembly are still unclear although both have been shown to be required for normal

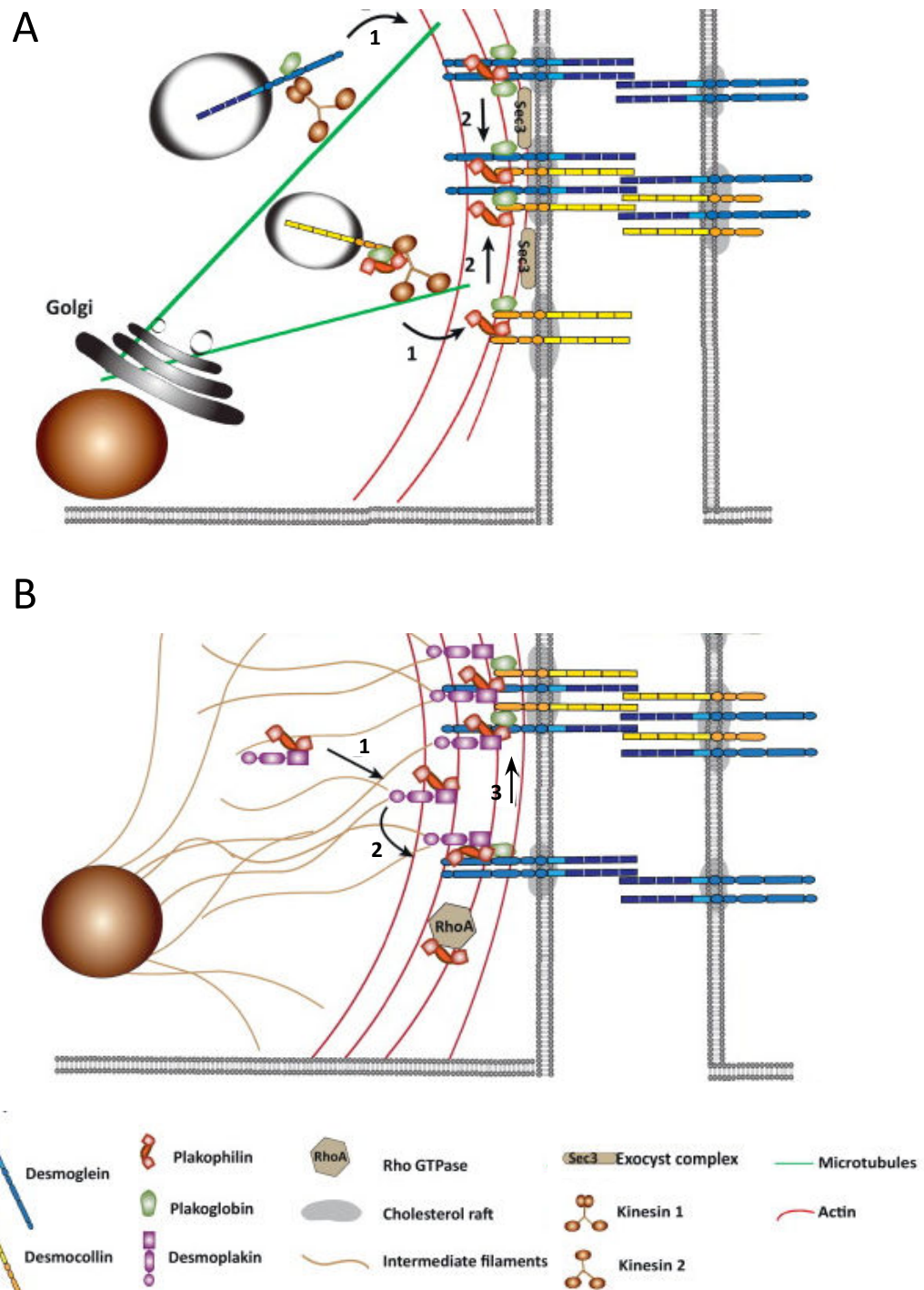


Figure 1.12 Model of Desmosome assembly. Illustrated in two phases, **(A)** illustrates the assembly of desmosomal cadherins at the plasma membrane, during this phase desmocollins- followed by desmogleins- containing vesicles are transported from the golgi along microtubules in a kinesin-2 and kinesin-1 dependent manner (1). Once at the plasma membrane they are incorporated into desmosomes a process facilitated by Secs3 (2). **(B)** Illustrates cytoplasmic plaque assembly. Initiated during Dsg3 membrane recruitment during which Dp is also recruited, Dp:Pkp enriched particles are transported to the plasma membrane via IF (1) where it is translocated to cell-cell contacts (2). Once at contact Dp binds associated and bind desmosomal cadherins via Pg to from the IDP (3). (Figure from Nekrasova and Green, 2013)

desmosome plaque assembly (Acehan, et al., 2008, Grossmann, et al., 2004, McGrath, et al., 1997, Ruiz, et al., 1996). For a more detailed description of desmosome assembly see Nekrasova and Green, 2013.

'Hyper' and 'hypo' adhesion

Desmosome exists in two adhesive states, calcium-dependent (*hypo-adhesive*) and calcium-independent (*hyper-adhesive*). During assembly and before desmosome stabilisation, desmosomes are calcium-dependent. At this stage desmosome adhesion can be dissociated by extracellular calcium depletion. After desmosome formation, a state of hyper-adhesion is gradually achieved allowing the structure to carry out its principle function in maintaining tissue integrity (Garrod, et al., 2005, Kimura, et al., 2007). In this state desmosomes can no longer be dissociated by calcium depletion (Garrod, et al., 2005, Kimura, et al., 2007). *In vitro*, hyper-adhesion of desmosomes is achieved through the maintenance of confluent culture for a few days and occurs, as currently understood, without altering molecular composition of the desmosome, but rather through the molecular re-arrangement of desmosomal cadherins through the entrapment of Ca^{2+} ions (Garrod, et al., 2005, Kimura, et al., 2007).

The adhesive state of desmosomes in early passage culture is reversible but ultimately replaced by calcium-independence (hyper-adhesiveness). During wound healing, desmosomes shift to a calcium-dependent state in order to facilitate cell migration and re-epithelisation. The ability of desmosomes to modulate their adhesive state is essential as they need to be as dynamic as the tissues they support. *In vitro* studies have shown that activation of PKC α causes reversion of desmosomes to calcium-dependence to facilitate epithelial remodelling in wound healing (Garrod, et al., 2005, Wallis, et al., 2000).

Regulation

Whilst being essential in providing mechanical strength, desmosomes themselves are dynamic structures with changes in their molecular composition and structure during processes such as epithelial differentiation and stratification, tissue remodelling and wound healing (Green, et al., 2010). Desmosomes are regulated at both transcriptional and post-transcriptional levels. Transcriptionally, understanding of how desmosomes are regulated is still limited. However, preliminary studies based on the characterisation of desmosomal cadherin promoters revealed that desmosomal cadherins are regulated by different promoters and the specific expression of each cadherin can be achieved without affecting the others (Denning, et al., 1998, Hunt, et al., 1999, Smith, et al., 2004). On a post-transcriptional level, signals generated by cell-cell contacts and junction assembly provoke protein phosphorylations that in turn positively and negatively regulate desmosome formation (Pasdar, et al., 1995). It has been shown that phosphorylation of serine residues of Dscs promotes Pg association and Dsg binding to confer desmosome formation in response to calcium (Aoyama, et al., 2009). PKC α has been demonstrated to have a pivotal role in the regulation of desmosomes, with its activation able to promote desmosome formation in a calcium- and adherens junction-independent manner (Sheu, et al., 1989, van Hengel, et al., 1997). PKC α has also been reported to be involved in Dp trafficking for its junctional incorporation through a Pkp2-dependent mechanism (Bass-Zubek, et al., 2008). Furthermore, it has been shown that the activity of PKC α is directly associated with the adhesive state of desmosomes with its suppression rendering desmosomes in a status of hyper-adhesion, whereas PKC α activation promotes calcium-dependence (Garrod, et al., 2005).

Both intracellular and extracellular calcium are required for desmosome junction formation and the stability of desmosomal components (Pasdar, et al., 1995, Pasdar and Nelson, 1988, Pasdar and Nelson, 1988, Pasdar and Nelson, 1989). Finally, desmosome assembly is dependent on adherens junction formation (Amagai, et al., 1995, Fujimori and Takeichi, 1993, Jensen, et al., 1997, Lewis, et al., 1994), with the relationship between these junctions attributed to Pg (Lewis, et al., 1997). However it has been shown that desmosomes formation can exhibit adherens junction independence (van Hengel, et al., 1997).

Table 1.1 Human desmosome-associated diseases. This table illustrates some of the human disease attributed to genetic, autoimmune or bacterial toxin factors mediating alteration of specific desmosomal components. Table adapted from (Green and Gaudry, 2000, Green and Simpson, 2007).

Desmosomal component	Human disease	References
Dsg 1 (genetic alteration)	Striate palmoplantar keratoderma (SPPK)	(Kljuic, et al., 2003, Rickman, et al., 1999)
Dsg 2 (genetic alteration)	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	(Pilichou, et al., 2006)
Dsg 3 (autoantibodies)	Pemphigus Vulgaris (mucosal dominant), Suprabasal epidermal acantholysis	(Amagai, et al., 1991, Lever, 1953)
Dsg 4 (genetic alteration)	Hypotrichosis	(Kljuic, et al., 2003)
Dsc 2 (genetic alteration)	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	(Heuser, et al., 2006, Syrris, et al., 2006)
Plakoglobin (genetic alteration)	Arrhythmogenic right ventricular cardiomyopathy (ARVC), Palmoplantar keratoderma	(McKoy, et al., 2000)
Desmoplakin (genetic alteration)	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	(Rampazzo, et al., 2002)
Plakophilin 1 (genetic alteration)	Ectodermal dysplasia and skin fragility syndrome	(McGrath, et al., 1997)
Plakophilin 2 (genetic alteration)	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	(Gerull, et al., 2004)
Dsg 1 (auto-antibodies)	Pemphigus foliaceus, Superficial and suprabasal epidermal Acantholysis, Pemphigus vulgaris (mucocutaneous), Mucosal erosions.	(Amagai, et al., 1991, Koulu, et al., 1984, Lever, 1953)
Dsg 1 (Bacterial toxins)	Staphylococcal scalded skin syndrome	<i>First described by Ritter von Rittershain 1878</i>

1.5.3 Desmosomes in disease

The functional importance of desmosomes and their constitutive molecular components in cell-cell adhesion is highlighted by a variety of human diseases which arise when desmosomal protein function is compromised. As illustrated in Table 1.1 predominantly the heart and skin, which are subjected to routine and substantial mechanical forces, are adversely affecting by a loss of desmosomal integrity and function. Interestingly, a variety of clinical presentations arise when different components are compromised, suggesting that whilst desmosomal components contribute to a shared common adhesive function, they also possess specific or unique functions in ensuring desmosomal integrity.

1.5.4 Desmosomal cadherin: Desmoglein 3

Desmoglein 3 (Dsg3), a 130 kDa protein, belongs to the desmoglein subfamily. As illustrated in Figure 1.5, Dsg3 exhibits tissue- and differentiation-specific distribution patterns, predominantly expressed in the basal and immediate supra-basal layers of the epidermis and throughout the entire squamous epithelium of the oral mucosa. Dsg3 is also known to be expressed in cardiomyocytes, brain arachnoid cells and the dendritic reticulum cells of lymph nodes (Schwarz, et al., 1990). The functional significance of Dsg3 in mediating cell-cell adhesion in desmosomes is demonstrated by the autoimmune blistering disease, Pemphigus Vulgaris (PV), where circulating auto-antibodies bind to Dsg3 in epithelia and oral mucosa, disrupting cell-cell adhesion.

Role of Dsg3 in epidermal proliferation, differentiation and morphogenesis

The tissue-specific expression profiles of desmosomal cadherins (Figure 1.11 and 1.13) have been shown by various research groups to serve a purpose. Dsg3 is predominantly expressed in proliferating epithelial cells, hence its restricted distribution in the epidermis (Mannan, et al., 2011). It has also been demonstrated in transgenic mice that the distribution of desmoglein isoforms regulates the structure and function of the epithelia. One such study by Elias *et al.* showed that by altering the expression profile of Dsg3 in stratified epithelia to that similar to the oral mucosa caused the epidermal tissue to exhibit histological and phenotypic characteristics resembling that of mucosal epithelium (Elias, et al., 2001). In line with this study, mis-expression of Dsg3 in the supra-basal layers of the epidermis resulted in abnormal hyper-proliferation and differentiation (Merritt, et al., 2002). Furthermore, a study using blocking peptides targeting the CAR site of desmosomal cadherins inhibited alveolar morphogenesis and positioning of mammary epithelial cells (Runswick, et al., 2001). It has been shown that the protein kinase C (PKC) isoenzymes antagonistically regulate differentiation-dependent expression of desmosomal cadherins in human keratinocytes. For instance, the differentiation-promoting novel PKC δ promotes Dsg1 expression while the growth-promoting novel PKC ϵ decreased its expression. In the same way, the expression of Dsg3 is regulated by these two Ca-independent PKC isoenzymes (Szegedi, et al., 2009). Taken together these studies suggest that the expression of desmosomal cadherins including Dsg3 is associated with epithelial proliferation, differentiation and homeostasis.

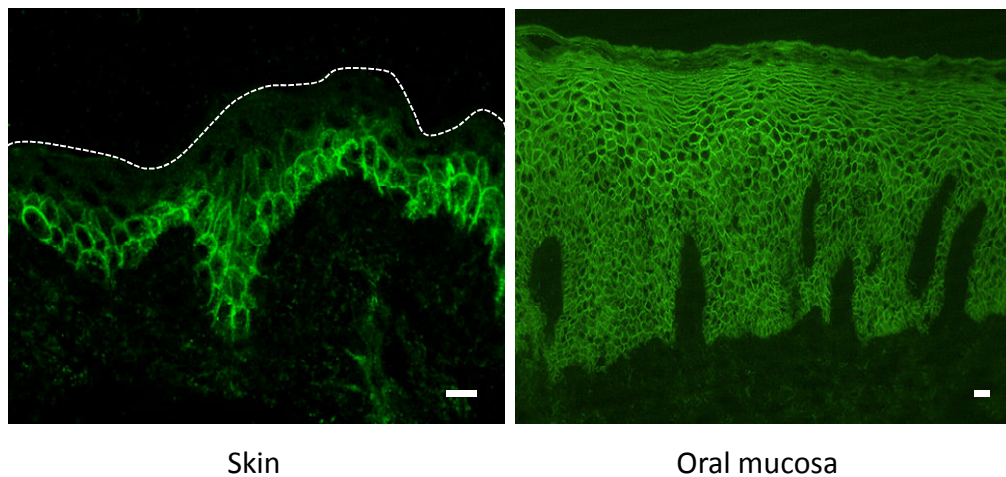


Figure 1.13. Desmoglein 3 expression in skin and oral mucosa. Tissue sections of human skin and oral mucosa were probed with Dsg3 specific antibody (green). Desmoglein 3 is expressed in the basal and immediate supra-basal layers of the epidermis (dotted line illustrates were the Stratum corneum ends) and throughout the entire stratified epithelium of oral mucosa (courtesy of Dr Hong Wan). Scale bar, 50µm.

Role of Dsg3 in the pathogenesis of pemphigus vulgaris

In 1991, Dsg3 was identified as the autoantigen in PV (Amagai, et al., 1991). PV is a rare autoimmune disorder characterised by the loss of keratinocyte adhesion within the spinous layer of the epidermis, in a process called ‘acantholysis’, which clinically manifests as blistering of the skin and oral mucosa. There are two subtypes of PV, mucocutaneous PV where autoantibodies targeting both Dsg3 and Dsg1 are produced, and mucosal dominant PV where autoantibodies against Dsg3 are circulating. Two additional types of pemphigus have also been characterised, Pemphigus Foliaceus (PF) in which autoantibodies target Dsg1 and Paraneoplastic Pemphigus (PNP) where autoantibodies target desmosomal and hemidesmosomal proteins (Anhalt, 2004). Pemphigus vulgaris is the most common form of pemphigus accounting for up to 80% of pemphigus cases with PF and PNP being less servers and least common, respectively. Histological examination shows that in PV blisters are largely restricted to the basal and immediate suprabasal layers of the epidermis and/or throughout the oral mucosa, in contrast blisters are restricted to the superficial layer of the epidermis with no mucosal involvement in PF. Despite many studies, the molecular mechanism of blister formation in PV remains unclear.

Pemphigus pathogenesis

Several hypotheses have been proposed to explain the mechanisms by which pemphigus manifests. Since the discovery of circulating auto-antibodies targeting Dsg3 and Dsg1 in the sera of PV patients, it was proposed that these auto-antibodies mechanically inhibit the adhesive function of Dsg1 and Dsg3 giving rise to the 'steric hindrance' hypothesis'. Other hypotheses include the 'desmoglein compensation' hypothesis (Brennan, et al., 2010) which attempts to explain the different clinical phenotypes between PV and PF. In accordance, Dsg3 is proposed to compensate, where possible, for the loss of Dsg1 and vice-versa. In the case of PF where Dsg1-mediated adhesion is compromised, the uniform expression of Dsg3 throughout the oral mucosa compensates its loss-of-function. However, in the epidermis where Dsg3 expression is limited to the basal layers of the epidermis; it is unable to compensate for the loss of Dsg1 in the stratum granulosum, as a result blister formation is limited to the superficial layers of the epidermis with no mucosal involvement. In PV where auto antibodies result in the loss of Dsg3-mediated adhesion, Dsg1 is unable to compensate for the loss of Dsg3-mediated adhesion throughout the oral mucosa resulting in mucosal dominant PV. Where auto-antibodies to both Dsg1 and Dsg3 are in circulation neither is able to compensate resulting in both epidermal and mucosal involvement, hence muco-cutaneous PV. Although a large body of evidence supports the 'desmoglein compensation' hypothesis clinical studies have found that the autoantibody titres do not always correlate with the clinical presentation, suggesting other factors, such as intracellular signalling, may play a role in the pathogenesis of pemphigus acantholysis.

Signalling hypothesis

Studies using anti-Dsg3 auto-antibodies to elucidate the possible signalling mechanism of pemphigus acantholysis, have uncovered overwhelming evidence of the ability of Dsg3 to initiate signalling cascades. The addition of such anti-bodies to keratinocyte culture has been shown to promote the re-organisation of cortical actin filaments (Berkowitz, et al., 2005, Gliem, et al., 2010), induce the phosphorylation of Dsg3 and its dissociation from desmosomes (Aoyama and Kitajima, 1999, Aoyama, et al., 1999, Yamamoto, et al., 2007), increase intracellular calcium concentrations (Kitajima, et al., 1999) and activate various signalling molecules such as Pg, PKC, p38 MAPK, heat shock

protein p27, Src and c-Myc (Berkowitz, et al., 2005, Kawasaki, et al., 2006, Spindler and Waschke, 2011, Waschke, et al., 2006). Taken together these findings not only affirm the signalling capabilities of Dsg3 but strongly suggest a signalling component in PV pathogenesis.

Role of Dsg3 in cancer

Traditional views consider desmosomes, and other intercellular junctions, as having a protective function in cancer through cell-adhesion-mediated contact inhibition. In accordance, the loss of cell-cell adhesion is an essential event in Epithelial to Mesenchymal Transition (EMT) (see section 1.1.2). In-keeping with this proposal, loss of desmosomes and their constitutive proteins have been found in various tumours in the body. EMT is a biologic process that transforms polarized epithelial cells through multiple morphological and biochemical changes to become mesenchymal in phenotype with characteristics such as, enhanced migration and invasion capability, resistance to apoptosis and increased production of extracellular matrix (ECM) components. Emerging evidence suggests that EMT is a prerequisite to pathological processes including cancer progression and metastasis. Loss of cadherin junctions and desmosomes occurs in epithelial cells to allow cellular dissociation in the conversion from benign to metastatic tumours (Chidgey and Dawson, 2007).

Paradoxically, several independent studies have shown Dsg3 to be up regulated in squamous cell carcinoma (SCC) of the head and neck, oesophageal and lung (Chen, et al., 2007, Fang, et al., 2014, Fukuoka, et al., 2007, Huang, et al., 2010) as well as cancer cell lines derived from head and neck SCC (Chen, et al., 2007). Furthermore, the level of overexpression correlating with clinical stage of disease and regional lymph node metastasis (Chen, et al., 2007, Fang, et al., 2014, Ferris, et al., 2005, Ferris, et al., 2011, Huang, et al., 2010). Dsg3 silencing suppressed cancer cell growth, migration and invasion *in vitro* and *in vivo* (Chen, et al., 2007). Analysis of clinical samples and cancer cell lines in a recent patent application for the use of Dgs3 in the diagnosis of cancer, report elevated Dsg3 expression in cancers of the colon, oesophagus, stomach, pancreas and skin (Aburatani, et al., 2013). Finally Dsg3 has been identified as an accurate biomarker for the detection of metastatic spread of SCC (Ferris, et al., 2005, Ferris, et al., 2011, Patel, et al., 2013, Wang, et al., 2007) and ancillary marker to separate SCC from other subtypes of lung cancer (Savci-Heijink, et al., 2009).

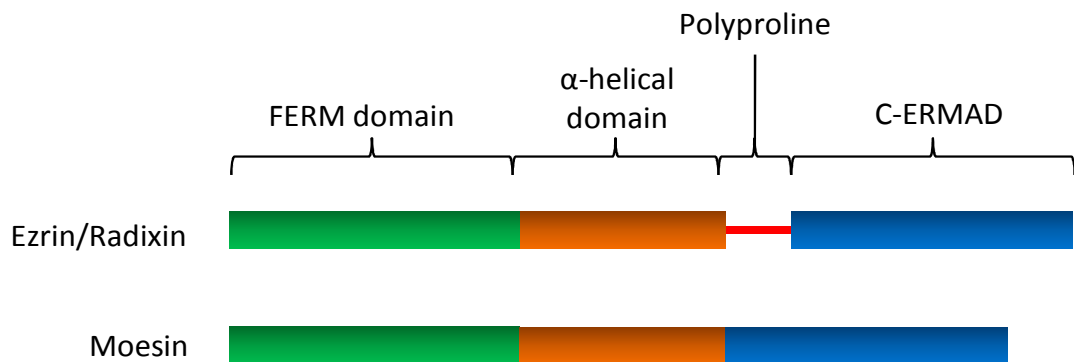


Figure 1.14 Structure of ERM family proteins. Ezrin, Radixin and Moesin share a common globular FERM domain at their N-terminus which is composed of three sub-domains (F1, F2 and F3) arranged in a cloverleaf configuration to confer membrane binding. The central domain form a monomeric α -helical rod which links the FERM domain to the F-actin binding domain at the C-terminal ERM associated domain C-ERMAD. The charged C-ERMAD contains a conserved sequence motif for the actin binding. Ezrin and Radixin contain an additional polyproline region which is absent in Moesin.

Collectively, these findings indicate a positive role for Dsg3 in cancer, however, little is known about its actual function and mechanisms by which Dsg3 regulates cancer cell growth and invasion.

1.6 ERM family proteins

Ezrin, Radixin and Moesin make up the ERM family proteins that belong to the band 4.1 superfamily, and function as regulated molecular linker proteins between the plasma membrane and the actin cytoskeleton (Arpin, et al., 1994, Bretscher, et al., 1997, Tsukita and Yonemura, 1997, Vaheri, et al., 1997). In doing so they have been implicated in the regulation of the actin cytoskeleton and actin based processes, such as membrane projections and dynamics (Bretscher, 1999, Bretscher, et al., 2002, Louvet-Vallée, 2000, Mangeat, et al., 1999, Tsukita and Yonemura, 1997), epithelial morphogenesis, cell adhesion and migration (Arpin, et al., 2011, Bretscher, et al., 2002, Fehon, et al., 2010).

1.6.1 Structure of ERM proteins

Figure 1.14 illustrates the structure of the ERM family proteins, characterised by the presence of a ~300 amino acid FERM (Four.one Ezrin, Radixin and Moesin) domain at the N-terminus followed by a α -helical domain capped by a C-terminal ERM associated domain (C-ERMAD) (Algrain, et al., 1993, Gary and Bretscher, 1995, Turunen, et al., 1994). The FERM domain is composed of three subdomains arrange in a clover-like

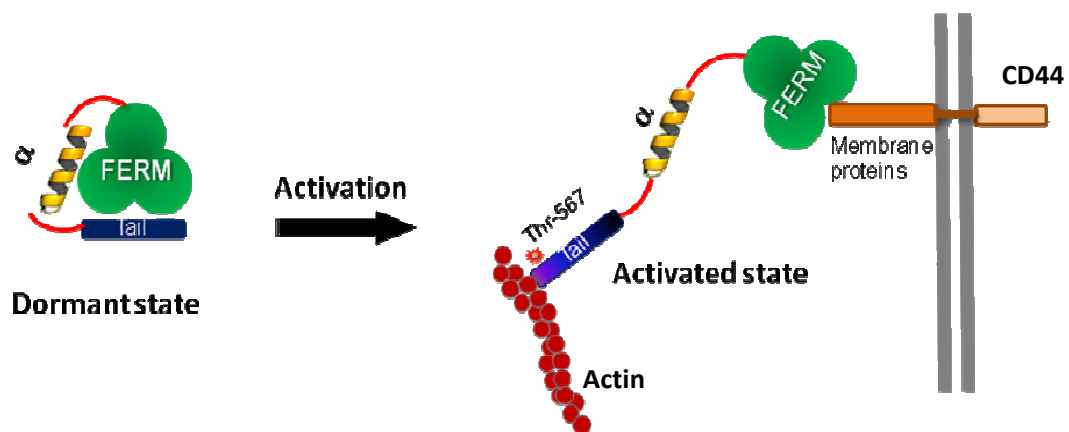


Figure 1.7 Model of the ERM protein activation. In a dormant state C-/N-terminus interaction masks the plasma membrane and F-actin binding sites of the molecule. The activation of the ERM proteins involves their recruitment to the plasma membrane and association with phosphatidylinositol 4,5-bisphosphate where they are subsequently phosphorylated at a conserved threonine residue which provokes a conformational change thus unmasking F-actin and plasma membrane binding sites.

shape and confers binding to the plasma membrane through direct association with integral membrane proteins or indirectly through membrane associated proteins. The C-ERMAD contains the actin binding site mediating the link between the plasma membrane and the actin cytoskeleton (Berryman, et al., 1993, Pearson, et al., 2000). The C-ERMAD can also associate with the FERM domain through self association or with other ERM molecules to form either homo- or hetero-dimers (Gary and Bretscher, 1995). This C-/N-terminal association masks the plasma membrane and actin binding sites rendering the protein inactive (Bretscher, et al., 2002, Gary and Bretscher, 1995).

1.6.2 Activation of ERM proteins

The activity of Ezrin, Radixin and Moesin is regulated by their conformational state. In the inactive state, an intra- or inter-molecular interaction between the C- and N-terminal domains masks the actin and membrane binding sites (Berryman, et al., 1995, Bretscher, et al., 1995, Gary and Bretscher, 1993, Gary and Bretscher, 1995, Magendantz, et al., 1995). In this inactive conformation the protein adopts a diffused cytoplasmic localisation (Hirao, et al., 1996, Takeuchi, et al., 1994). Upon activation ERM proteins unfold revealing their binding sites for the plasma membrane and cortical actin. As currently understood, activation is achieved in a 2-step model where inactive ERM proteins are targeted to regions of the plasma membrane rich in phosphatidylinositol 4,5 bis-phosphate (PIP₂) to which they bind via their FERM domain

to provoke a conformational change (Fievet, et al., 2004, Nakamura, et al., 1999, Yonemura, et al., 2002) unmaking the C- and N-terminus binding sites. This event is followed by the phosphorylation of a conserved C-terminal threonine residue T567, T564 and T558 for Ezrin, Radixin and Moesin respectively, located at the C-ERMAD domain (Fievet et al. 2004, Zhu et al. 2007). Studies have found that the serine/threonine kinases responsible for this phosphorylation event include PKC α and θ , NIK (NF κ B-inducing kinase), LOK (Lymphocyte-Orientated Kinase) and MST4 (Baumgartner, et al., 2006, Belkina, et al., 2009, Ng, et al., 2001, Pietromonaco, et al., 1998). As illustrated in Figure 1.7, In the open confirmation the N-terminal FERM domain of Ezrin interacts and binds to the plasma membrane directly through integral proteins such as CD44 or ICAM2 (Yonemura, et al., 1998), or indirectly through cytoplasmic membrane associated proteins, such as EBP50 and E3KARP via their PDZ domains (Reczek, et al., 1997). The C-terminal domain of Ezrin interact and bind to cortical F-actin.

1.6.3 Function of ERM proteins

Co-expressed and colocalised in most cultured cells, ERM proteins are found concentrated at membrane associated structures such as microvilli, ruffles, cell-cell contacts and cell-matrix adhesion sites where actin cytoskeleton is densely associated with the plasma membrane (Arpin, et al., 1994, Takeuchi, et al., 1994). The ERM proteins exhibit tissue- and cell type-specific expression and distribution patterns with Ezrin most abundant in epithelial cells, Radixin in hepatocytes and Moesin in endothelial cells. Irrespective of their specific expression and distribution, the commonality of their principle associations with the actin cytoskeleton and plasma membrane, unite them in regulating and organisation of actin-rich plasma membrane domains (Bretscher, 1989, Takeuchi, et al., 1994). The specific function of ERM proteins is dictated by the ERM protein involved, the mode of phosphorylation and phosphorylation site, a selection of which are illustrated in Table 1.2.

1.6.4 Functional redundancy of ERM proteins

The similarities in ERM protein expression, localisation and binding affinities for the plasma membrane (Hirao, et al., 1996, Turunen, et al., 1994) and F-actin in addition to a high level of amino acid identity (>70%), favours the idea that ERM proteins exhibit

Table 1.2 Specific functions of ERM family proteins with respect to phospho-site and mode of phosphorylation. Adapted from (Clucas and Valderrama, 2014)

ERM protein	Phosphorylation site	Kinase involved	Function of activated ERM
Ezrin	Thr567	Akt2	NHE3 translocation and activation
	Thr235, T567	CDK5	Cell senescence
	Thr567	LOK	Cytoskeleton rearrangement in hematopoietic cells
	Thr567, Tyr145, Try353	EGFR	Cell proliferation, lamellipodia formation
	Tyr145, Tyr477	Src	Cell spreading
	Thr567	SLK	Microvilli formation in polarised epithelial cells
	Thr567	ROCK	Effector of RhoA
	Thr567	PKC	Cell migration
	Ser66	PKA	Gastric parietal cell activation
Radixin	Thr564	NIK	Lamellipodia formation and cell morphogenesis
	Thr564	PKC	Cell migration
	Thr 564	ROCK	Fas-mediated apoptosis
Moesin	Thr588	LOK	Microvilli formation in polarised epithelial cells
	Thr588	NIK	Lamellipodia formation and cell morphogenesis
	Thr558	PKC	Cell migration
	Thr588	ROCK	Cell migration and invasion
	Thr588	MRCK	Effector of Cdc42 in filopodia formation

functional redundancy. Early *in vivo* studies to elucidate specific ERM protein function showed that the individual silencing of ERM proteins brought about no significant phenotypic affect with only the combined suppression of all three ERM proteins resulting in the destruction of cell-cell and cell-matrix adhesion and concomitant disappearance of microvilli in mouse epithelial and thymoma cells (Takeuchi, et al., 1994) . Later studies suggest that ERM proteins are able to compensate for one another (Doi, et al., 1999, Kikuchi, et al., 2002, Saotome, et al., 2004). These reportssupport the concept of functional redundancy amidst ERM family proteins. However, their structural differences, cell-specific expression patterns and specific protein functions suggest functional diversity. Several research groups are dedicated to uncovering the unique functions of ERM proteins and have been successful in

identifying tissue specificity, unique features such as exclusive phosphorylation sites (Takeuchi, et al., 1994) and specific functions (Table 1.3). Thus, when attempting to ascertain individual functions related to any one member of the ERM family proteins, caution and consideration for possible compensatory events should be taken when drawing conclusions.

1.6.5 Ezrin

As the most extensively studied ERM protein, Ezrin has provided the basis of our understanding of ERM protein function. Ezrin is an 81 kDa protein and, like other ERM proteins, functions as a regulated linker protein. As shown in Figure 1.16, in its active conformation, the FERM domain of Ezrin is able to associate directly with the plasma membrane through binding to integral proteins including CD44 (Hirao, et al., 1996, Tsukita, et al., 1994), ICAM-2 (Yonemura, et al., 1998), PA2.26 (Scholl, et al., 1999) and syndecan 2 (Granés, et al., 2000) or indirectly through membrane associated proteins including EBP50 (Ezrin binding phospho-protein 50) and E3KARP (Bretscher, et al., 1997, Reczek, et al., 1997). Other residues including Thr235 (Yang and Hinds, 2003), Tyr145 and Tyr353 (Krieg and Hunter, 1992) have been shown to be phosphorylated (Krieg and Hunter, 1992), associated with functions such as, cell proliferation, senescence and spreading (Table 1.3).

Role of Ezrin in cell morphology, adhesion, migration and apoptosis

Beyond acting as a membrane cytoskeleton linker protein, functional analysis of Ezrin has demonstrated significant effects on actin cytoskeletal organisation with the formation of numerous cellular projections in insect Sf9 or CHO cells overexpressing the truncated C-terminus of ezrin (Martin, et al., 1995). Likewise the expression of Ezrin T567D mutant (permanent phospho-mimic) caused elongated projections of the plasma membrane of cultured gastric parietal cells (Zhou, et al., 2005). Knockdown studies support the role of Ezrin in the formation and maintenance of actin rich structures. *In vivo* studies performed by Takeuchi *et al.* who showed that the suppression of all ERM protein resulted in the disappearance of microvilli in epithelial cells (Takeuchi, et al., 1994), while the ablation of Ezrin at the tips of membrane extensions in Rat-1 fibroblasts, results in membrane retraction (Lamb, et al., 1997). With respect to cell migration, the inactivation of the cytoplasmic pool of Ezrin results

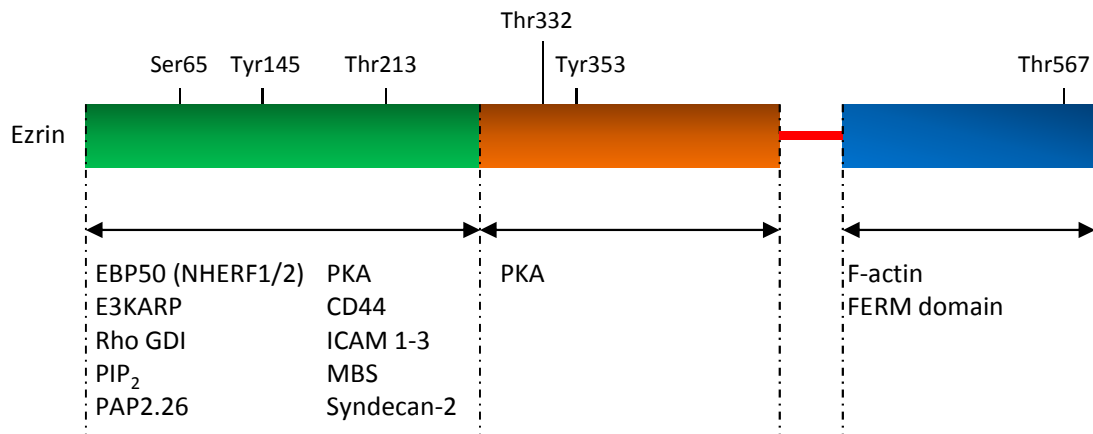


Figure 1.16 Structure of Ezrin including binding sites, phospho-sites and protein associations. Illustrated is the domain organisation of Ezrin that is composed of a N-terminal FERM domain followed by a α -helical domain, a polyproline rich linked by a C-terminal C-ERMAD domain. Also indicated are the identified residues for the phosphorylation and the particular domains for protein interaction.

in retraction of the plasma membrane from the leading edge in Rat-1 cells lines. Another study carried out by Crepaldi *et al.* showed that Ezrin is able to regulate cell migration induced by HGF stimulation in LLC-PK1 cells (Bretscher, 1989, Crepaldi, et al., 1997). Similarly PKC α dependent phosphorylation of Ezrin-T567 promotes cell migration, while the Ezrin mutant T567A impairs PKC α mediated motility in the MCF-7 breast carcinoma cell line (Ng, et al., 2001).

Ezrin has also been shown to enhance cell adhesion (Kaul, et al., 1996, Martin, et al., 1995) as knockdown of Ezrin inhibits both cell-cell and cell-substrate adhesion (Bretscher, et al., 1997, Hiscox and Jiang, 1999, Takeuchi, et al., 1994). Studies have also implicated Ezrin in cell survival through PI 3-Kinases/Akt pathway in epithelial cells. In these experiments they demonstrated that Ezrin interacts with p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), at two distinct sites to convey an anti-apoptotic signal (Gautreau, et al., 1999). Finally, Zhu *et al.* demonstrated that the establishment of cell polarity is also dependent upon the Ezrin activity (Zhu, et al., 2010). Taken together, these findings suggest that Ezrin plays a role in multiple cellular processes including cell adhesion, morphology, migration and apoptosis.

Role of Ezrin function in Signal transduction

It has been shown that ERM family proteins associate with a variety of signalling molecules, close inspection reveals that they act as both upstream regulators and

downstream effectors of specific molecules. Several groups have shown that Ezrin activation is the event downstream of signalling by Rho and PKC, with Rho kinases and PKC responsible for the phosphorylation of the conserved C-terminal threonine residues of Ezrin (Matsui, et al., 1998, Shaw, et al., 1998) that renders the conformational change and mobilisation of ERM from cytoplasmic pool to the plasma membrane protrusions (Shaw, et al., 1998). Furthermore, Rho-dependent activation of ERM proteins is shown to be crucial for Rho-induced cytoskeleton rearrangements (Mackay, et al., 1997). In support, inhibition of Rho kinases inhibits the phosphorylation of Ezrin T567 in hepatocarcinoma and Jurkat cells (Chen, et al., 2011, Hébert, et al., 2008). Rho is also shown to function as a downstream effector of Ezrin as shown by Hatzoglou *et al.* In this study activated Ezrin binds Gem, to down-regulate RhoA signalling pathways through interactions with Gem-interacting protein (Gmip) (Hatzoglou, et al., 2007). Gem is a GTP-binding protein of the Ras superfamily which regulates cytoskeleton organisation and calcium channels (Maguire, et al., 1994). Gmip is a novel Rho-GTPase-activating protein (Rho-GAP) which functions, like other the GAPs, to increases the intrinsic GTPase activity of Ras-related proteins returning them to their GDP-bound state *i.e.* a state of inactivation or basal state (Aresta, et al., 2002).

Role of Ezrin in cancer progression

An Ezrin-dependent mechanism, through the activation of RhoA, has been shown to promote EMT (Martín-Villar, et al., 2006). Furthermore, Ezrin has been shown to be associated with cancer progression (Hunter, 2004, Khanna, et al., 2004, Ren, et al., 2009, Yeh, et al., 2009), dynamically up-regulated during tumour metastasis (Khanna, et al., 2004, Ren, et al., 2009). In particular, Ezrin is found to be transiently upregulated in early metastatic progression and again during metastatic expansion but down-regulated during the establishment and survival of metastatic nodules. Chen *et al.* demonstrated that the hyper-phosphorylation of Ezrin Thr567 correlates with an invasive phenotype in hepatocarcinoma, with the inhibition of invasion achieved by Rho kinase-dependent inhibition of Ezrin activity (Chen, et al., 2007). Taken together, this suggests that Ezrin plays an important role in cancer progression and metastasis.

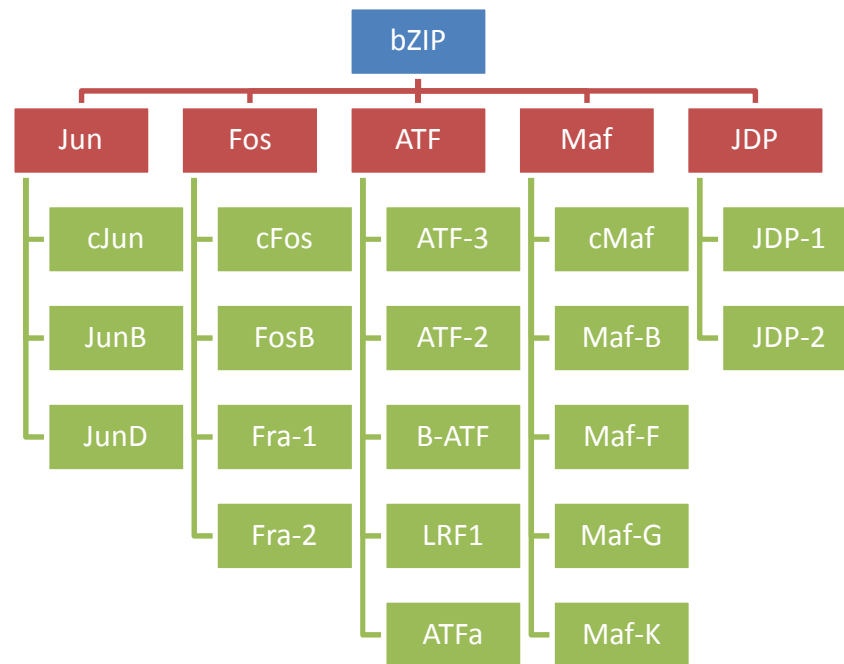


Figure 1.17 Activating Protein-1, bZip family proteins. Activating Protein-1 (AP-1) is composed of homo- and hetero-dimers of AP-1 bZIP proteins, including Jun, Fos, ATF, Maf and JDP subfamilies. The dimeric composition is determined by the abundance of the constituents and the varying affinities they have for each other.

1.7 Transcription factor: Activator Protein 1

As mentioned in section 1.3 several transcription factors have been identified as regulators in EMT such as the ZEB, Snail and Twist (Sánchez-Tilló, et al., 2012) and also play a central role in cancer progression such as GATA and E2F (Hollern, et al., 2014, Lopez-Bergami, et al., 2010, Zheng and Blobel, 2010). In both cases AP-1 has also been implicated (Davies, et al., 2005, Lopez-Bergami, et al., 2010), furthermore Ezrin is a known transcriptional target of AP-1 that confers invasiveness (Jooss and Müller, 1995) and AP-1 activity has been shown to be required for the correct localisation of Ezrin to the plasma membrane following EGF treatment (Stapleton, et al., 2002).

Activator protein 1 (AP-1), originally described as the TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) inducible transcription factor, is a dimeric complex that regulates gene transcription by binding to the TPA response elements (TRE, consensus sequence 5'-TGAG/CTCA-3') and the cAMP-response elements (CRE, consensus sequence 5'-TGACGTCA -3') located in the promoter and enhancer regions of AP-1 inducible genes. AP-1 has an expansive transcriptional repertoire propagated by its diverse homo- and hetero-dimeric compositional array of Jun, Atf, Fos and Maf bZIP protein families, a selection of which are illustrated in Figure 1.17. bZIP superfamily proteins are

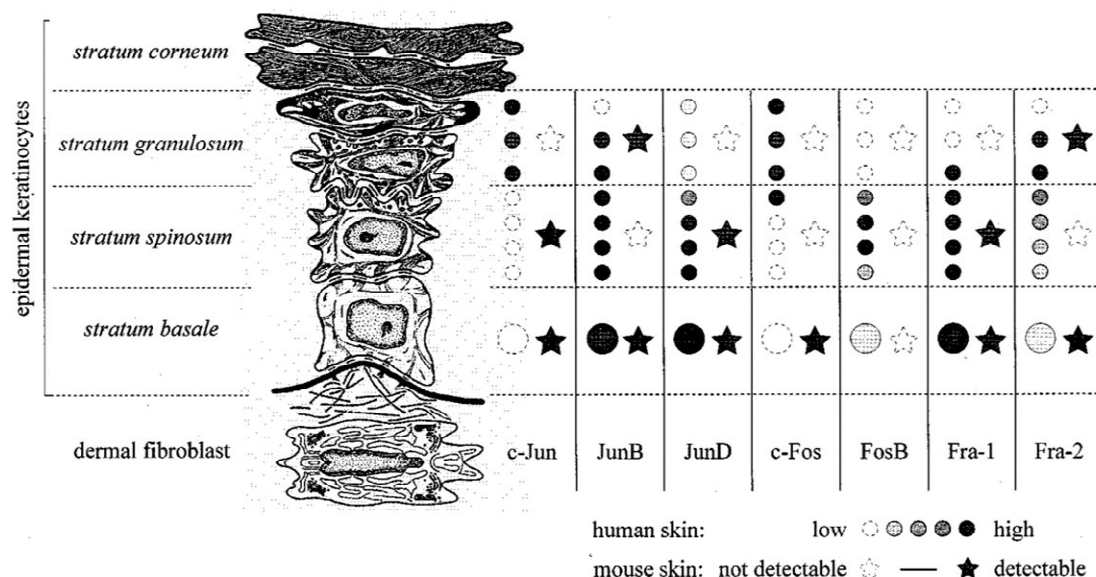


Figure 1.18 Expression patterns of AP-1 bZip proteins in human and mouse skin. As illustrated AP-1 subunit cJun, JunB, JunD, cFos, FosB, Fra-1 and Fra-2 are expressed in specific layers of the epidermis which differ in their state of keratinocyte differentiation (Adapted from (Angel, et al., 2001))

characterised by the presence of a C-terminal highly charged basic DNA binding domain and a dimerisation domain referred to as a 'Leucine zipper', in short referred to a bZIP (basic region leucine zipper) proteins. AP-1 has been shown to regulate a vast array of cellular processes including development, proliferation, apoptosis, immune responses, and stress responses (Foletta, et al., 1998, Shaulian and Karin, 2001, Wagner, 2002, Zhou, et al., 2001) Moreover, the deregulation of AP-1 has been associated with a variety of pathologies including inflammatory disease of the bone, skin and liver (Thomsen, et al., 2013, Zenz, et al., 2008) and upregulated in numerous malignancies, including skin breast, cervix and lung (Eckert, et al., 2013, Ouyang, et al., 2008, Prusty and Das, 2005, Shen, et al., 2008, Yu, et al., 2012).

1.7.1 Regulation of AP-1 activity

The diversity of the genes regulated by AP-1 is due to the complex regulatory mechanisms which either increase the abundance of AP-1 proteins or those that stimulate their activity. Through these mechanisms the state of AP-1 activation, bZIP protein abundance and expression patterns, translational and post translational modifications and interaction with ancillary proteins are modulated. Dimer composition is a powerful means of regulating AP-1 activity as DNA binding affinity,

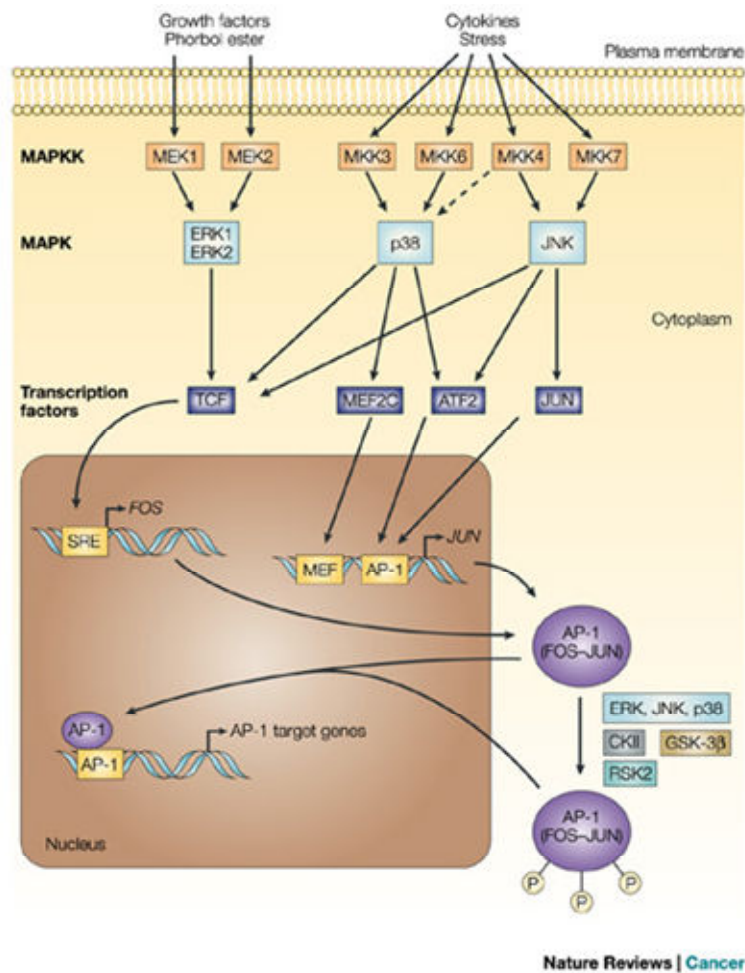


Figure 1.20 Overview of transcriptional and post-translational regulation of AP-1. As illustrated, the activation of MAPKs ERK1/2, p38 and JNK promotes the transcription of cFos and cJun. cFos:cJun AP-1 dimer can be further regulated through phosphorylation to promote transcription of AP-1 target genes (JNK, ERK, p38, RSK2) or inhibit transcription (GSK-3, CKII). Adapted from Eferl *et al.*, 2003.

AFT proteins are also able to homodimerise but Fos proteins cannot (Ziff, 1990). Dimer composition also has a direct effect on the DNA-binding site preference, as individual proteins exhibit response elements preferences (Eferl and Wagner, 2003), which when dimerised determines which element is bound and subsequently which genes are transcribed. Jun/Jun and Fos/Jun have been shown to bind with high affinity to TRE whereas Jun/ATF dimers and ATF homodimers bind to CRE (Hai and Curran, 1991). Additionally, some dimer combinations including Maf family proteins preferentially bind to other elements including MARE I, MARE II (MAF-recognition elements) and ARE (antioxidant element) (Eferl and Wagner, 2003). Transcriptionally, the abundance of AP1-components can be influenced by various extracellular signals transduced through

Table 1.4 Target genes of AP-1 implicated in cancer development and suppression.
Adapted from Eferl and Wagner *et al* and Ozanne *et al* (Eferl and Wagner, 2003, Ozanne, et al., 2007)

Process	AP-1 target genes
Oncogenic transformation	cJun, cFos
Angiogenesis	VEGF-D, uPA, uPAR, Proliferin
Apoptosis	Bcl ₂ , Bcl _{xl} , FasL Bcl ₂ , BIM, FAS, p53
Migration, invasive growth and metastasis	MMP-1, MMP-3, MMP9, uPA, Cd44, Ezrin, Cathepsin, MTS1,KRP1, TSC36/FRP, Tropomyosin 3, Tropomyosin 5b, ARP 2/3, CapG, CD44, Ezrin, SSeCKs,
Proliferation	p53, p21,p19, cyclin D1, EGFR, HB-EGF, GM-CSF,KGF, WAF1, INKA4

MAPK. For example, the activation of JNK in turn activates ternary complex factors (TCFs) to induce cFos expression (Cavigelli, et al., 1995) while activated p38 induces cJun expression through the activation of transcription factor MEF2C (Wei, et al., 2003). Post-transcriptionally, the activity of both pre-existing and newly synthesized AP-1 components can be achieved through phosphorylation. With respect to cJun, PKC-dependent phosphorylation of the N-terminal residues inhibits DNA binding of cJun homodimers but not cJun:cFos heterodimers (Boyle, et al., 1991). Whereas phosphorylation of cJun at serine 73 and, to a lesser extent, serine 63 by JNK enhances its ability to activate transcription (Smeal, et al., 1994) (Figure 1.19). The transcriptional and post-transcriptional regulation of AP-1 components is summarised in Figure 1.20. Phosphorylation of AP-1 components has also been shown to play a role in protein stability with the phosphorylation of amino-terminal serine/threonine residues of cJun to reduce its ubiquitination and subsequent degradation (Musti, et al., 1997). Finally, the activity of AP-1 is also regulated by its interaction with ancillary proteins and co-factors. Interactions of JNK phosphorylated cJun with co-factor CBP (CREB-binding protein) is proposed to confer an increase in cJun transcriptional activation (Bannister, et al., 1995, Kwok, et al., 1994). Interaction with JAB1 (Jun activating domain binding protein) with cJun:JunD stabilises the AP1 complex and enhances transcriptional activity (Claret, et al., 1996). Nuclear receptors including GR (glucocorticoid), and RAR (retinoic-acid receptor) and nuclear protein p202 inhibit AP-1 activity through trans-repression, a process of mutual negative interference between

transcription factors, though direct interaction, and the inhibition of DNA binding in the case of p202 (Benkoussa, et al., 2002, Min, et al., 1996, Schüle, et al., 1990, Yang-Yen, et al., 1990).

1.7.2 Role of AP-1 in Cancer

The major mammalian components of AP-1, cJun and cFos were originally identified as the viral oncoproteins vJun and vFos, shown to have positive effects on cell transformation. Today, other AP-1 proteins have been identified, some of which have also been associated with cancer progression whereas others identified as potential tumour suppressors. The pro- or anti-oncogenic functions of AP-1 in tumorigenesis is attributed to normal or aberrant regulation of target genes that influence cell proliferation, apoptosis, survival, invasive growth and angiogenesis (Table 1.4).

AP1 in tumour suppression

Some AP-1 components lack transforming ability such as the Jun family proteins, JunB and JunD. Interestingly, both these proteins as well as others have been identified as having tumour suppressor functions (Deng and Karin, 1993). Both JunB and JunD down-regulate processes of proliferation and/or stimulate apoptosis (Passequé, et al., 2001, Passequé and Wagner, 2000, Pfarr, et al., 1994, Shaulian, et al., 2000, Szabowski, et al., 2000). Furthermore, both *in vivo* and *in vitro* studies have shown that heterodimers containing JunB with oncogenic bZip proteins can antagonise oncogenic transformation (Chiu, et al., 1989, Passequé, et al., 2001). The ability of AP-1 to function as a tumour suppressor is still debateable as comparatively few studies have been conducted to investigate its anti-oncogenic function. However, protective function of AP-1 is believed to be dependent on cell type, state of differentiation, tumour stage and genetic background (Eferl and Wagner, 2003).

AP-1 in epithelial to mesenchymal progression

The process of EMT is an essential programme in processes including wound healing, development and the inflammatory response plays an essential role in cancer progression, with cancer cells using this programme to metastasise and colonise distal sites (Nieto, 2013) (see section 1.1.2). Various signalling pathways are involved in inducing EMT including TGF β , Integrin, RTKs and Wnt signalling via GSK, p38, JNK, ERK, and β catenin:TCF/LEF, (Lamouille, et al., 2014) all of which are implicated in the

Table 7.1 AP-1 components overexpressed in various cancers.

Cancer	AP-1 component(s)	reference
Breast	c-Jun,Fra1 c-Jun	(Baan, et al., 2010) (Liu, et al., 2002) (Sankpal, et al., 2011)
Ovarian	c-Jun	(Neyns, et al., 1999) (Hein, et al., 2009)
Endometrial	c-Fos, Fra2 and JunB c-Jun	(Bamberger, et al., 2001) (Alkhalaf, et al., 1993)
Hodgkins Lymphoma	AP-1 c-Jun, JunB	(Green, et al., 2012) (Mathas, et al., 2002)
Colorectal	c-Jun, JunB c-Jun	(Wang, et al., 2000) (Suto, et al., 2004)
Cervical	c-Fos:JunB c-Jun	(Prusty and Das, 2005) (Maritz, et al., 2011)
Lung and Bladder	AP-1	(Linardopoulos, et al., 1993)
Osteosarcoma	c-Jun:c-Jun c-Jun:c-Fos	(Papachristou, et al., 2003) (Leaner, et al., 2009) (Wang, et al., 1995)
Head and Neck	cFos AP-1	(Swenson, et al., 2011) (Chen, et al., 2008) (Ondrey, et al., 1999) (Bancroft, et al., 2002)

activation or regulation of AP-1 activity (Figure 1.12) (Davies, et al., 2005, Eferl and Wagner, 2003, Lamouille, et al., 2014).

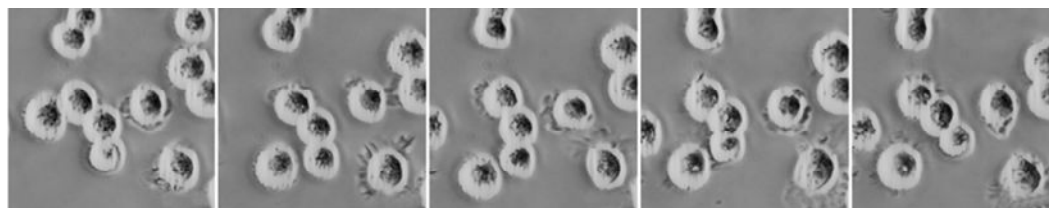
AP-1 in cancer progression

The role of AP-1 in cancer promotion is complex with aberrant expression of various components attributing to numerous cancers (Table 1.4). It has been shown that AP-1 is activated in response to oncogenic signalling transduction cascades as well as displaying oncogenic potential through its constituents, with the overexpression of a variety of bZip proteins including cJun, cFos and FosB able to transform cells efficiently (Hartl and Bister, 1995, Wang, et al., 1991). The transforming ability of these proteins is believed to be conferred by their potent transactivation potential (Jochum, et al., 2001). However, activating mutations in Jun have yet to be identified in human cancers (Lopez-Bergami, et al., 2010), therefore increased AP-1 activity and expression associated with cancer progression and metastasis is attributed to the constitutive up regulation of upstream Kinases (Lopez-Bergami, et al., 2010) such as ERK as a result of mutations in B-raf or N-ras (Davies, et al., 2002, Gorden, et al., 2003). Irrespective of the mode of increased AP-1 activity, the effect is the aberrant expression of AP-1

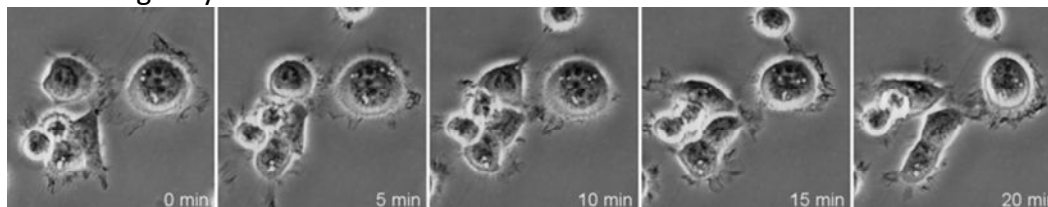
target genes that promote cancer progression and metastasis. As in normal physiology, the transcriptional array of AP-1 target genes in response to oncogenic activation will be governed by the dimeric composition of AP-1 which alters promoter binding specificity, transcriptional capacity and protein stability as outlined previously. This is then further influenced by the genetic background of the malignancy which will initially dictate the mode of oncogenic AP-1 activation, and furthermore by factors such as cell cycle progression and specific stimuli such as those from the tumour microenvironment (Lopez-Bergami, et al., 2010). To implicate a pivotal transcriptional regulator such as AP-1 a possible mechanism of Dsg3-induced cancer cell migration and invasion would strongly suggest that Dsg3 plays an active role in cancer progression through the regulation of the migration invasion programme.

A

A431 – Vector Control



A431 –Dsg3.myc



B

A431: EzrinDsg3DAPI

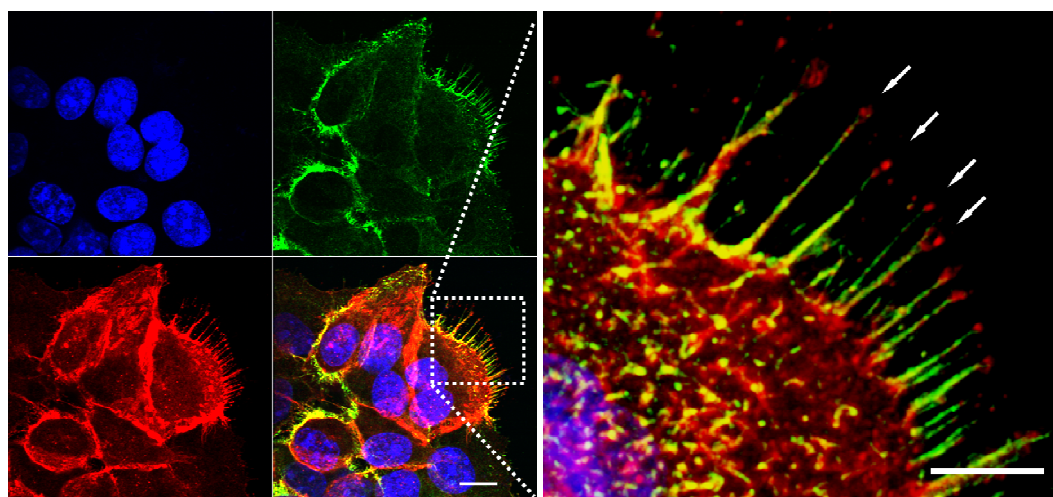


Figure 1.21 The overexpression of Dsg3 promotes membrane dynamics, cell spreading and membrane protrusion in A431 cells in which the colocalisation of Dsg3 and the membrane actin liker protein, Ezrin was shown. (A) Time lapse microscopic examination of A431 cells with transduction of full length human Dsg3 (hDsg3.myc) or empty vector (Vector control) that serves as a control. Image acquisition started immediately after plating cells. The overexpression of Dsg3 promoted cell spreading, membrane dynamics and protrusions compared to vector control. **(B)** Fluorescence confocal microscopy of A431-hDsg3.myc cells dual labelled with the Dsg3 (Red) and Ezrin (green) specific antibodies revealed the colocalisation of these two proteins, particularly at the plasma membrane and membrane protrusions (white arrows). Scale bars 10 μ m, Inset shows x5 magnification.

1.8 Preliminary data

In order to investigate the function of Dsg3 in cancer in detail, Dr Wan generated a stable A431 cell line overexpressing full length human Dsg3 (Tsang, et al., 2010). Preliminary analysis of these cell lines using time lapse and confocal microscopy revealed that the overexpression of Dsg3 in A431 cells (A431-hDsg3.myc) provoked pronounced membrane projections and dynamics, as well as enhanced cell spreading

compared to the vector control (Figure 1.21A) (Tsang, et al., 2010). Furthermore, fluorescent confocal microscopy of A431-hDsg3.myc cells immunostained for Dsg3 and Ezrin illustrated colocalisation of these two proteins, particularly at the plasma membrane and membrane projections (Figure 1.21B) (Tsang, et al., 2010).

Hypothesis and objectives.

Evidence that Dsg3 elicits function beyond the mediation of adhesion within desmosomes began with studies to elucidate the mechanism of PV implicating the ability of Dsg3 in the regulation of the actin cytoskeleton as well as induce intracellular signalling cascades. More recently Dsg3 has been identified, against conventional understanding, to be upregulated in a variety of cancer. Together there is a suggestion that Dsg3 may carry out functions outside of the desmosome and identifies an area which is under developed in literature. As discussed in this chapter, many studies have independently implicated both Dsg3 and Ezrin in oncogenesis with the upregulation of Dsg3 and Ezrin expression as well as increased Ezrin activity being associated with cancer progression. However a functional or mechanical link between these proteins has not been identified in literature. The preliminary observations above demonstrate that the overexpression of Dsg3 promotes actin dynamics and cell spreading which is consistent with the observed Dsg3-dependent actin re-organisation identified in PV studies. It is therefore possible, under specific circumstances that Dsg3-dependent actin regulation could promote cellular events such as cell migration, consistent with Dsg3-associated cancer cell behaviour. The preliminary findings further suggest a possible association between Dsg3 and established actin cytoskeleton regulator, Ezrin, which could theoretically facilitate Dsg3-dependent cellular events with respect to actin based processes, namely cell morphology, migration and invasion.

Hypothesis

With respect to current understanding and the presented preliminary findings, I propose that Dsg3 associates with Ezrin to co-operatively function in regulating the actin based processes that attribute to the positive role of Dsg3 in cancer progression and metastasis.

Aims of this study

To examine this hypothesis the aims of this study were as following: 1) to characterise the phenotype of cells overexpressing Dsg3; 2) to characterise the association between Dsg3 and Ezrin in A431 cell lines, and finally 3) to elucidate the underlying molecular mechanisms involved.

Chapter 2: **Materials and Methods.**

2.1 Cell culture

2.1.1 Cell lines and culture condition.

A431 cell line

Established by Giard and colleagues in 1973, the A431 cell line is a human cancer cell line derived from the vulva squamous cell carcinoma of a 85 year old female (Giard, et al., 1973). It expresses high levels of EGF receptor and harbours a loss-of-function p53 mutation. These cells are morphologically similar to normal differentiated squamous epithelial cells and exhibit reduced expression of junctional proteins (Atsumi, et al., 2008). In this study A431 cell lines were maintained in normal growth media, Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) supplemented with 10% foetal calf serum (FCS) (Biosera, UK). Media were changed every 3 days to maintain cell cultures.

SqCC/Y1 cell line

SqCC/Y1 cells are a human head and neck cancer cell line derived from buccal squamous cell carcinoma. These cells were maintained in normal growth media, EpiLife (Gibco) supplemented with EpiLife Defined growth supplement (Gibco). Media was changed every 3-4 days to maintain cell cultures. All cell lines were maintained in a humidified atmosphere at 37°C and 10% CO₂ in tissue culture incubators.

Cell passage

Once cell lines reached a density of 80% confluence, cells were split in a 1:10 ratio. To passage cells, the growth media were removed and replaced with 2 ml, 5 ml or 8 ml of trypsin/EDTA (Lonza, UK) for cells grown in T25, T75 and T175 tissue culture flasks, respectively. Cells were incubated with trypsin until completely detached from the flask, this would take approximately 5-10 min for SqCC/Y1 and 10-15 min for A431 cells. Once detached, an equal quantity of growth media containing serum was added to the trypsin:cell suspension. The serum was required to deactivate the proteolytic activity of trypsin. The cell suspension was then transferred to a conical centrifuge tube and centrifuged at 800 rpm for 5 min to pellet cells. The supernatant was carefully removed without disturbing the pellet. Cells were then re-suspended in fresh growth media supplemented with 10% serum. An aliquot of the cell suspension (10%) was seeded into a new flask containing growth medium.

2.1.2 Cryopreservation and recovery of cell stocks

Cells for cryopreservation were grown to 70% confluence before passaging as described above. However, in this case cell pellets were re-suspended in Freeze down media (FDM) (10% DMSO in FSC). For T25, T75 and T175 tissue culture flasks, pellets were re-suspended with 4 ml, 8 ml and 12 ml of FDM, respectively. Aliquots of 1ml were then transferred to 2.5 ml cryovials and frozen down at -80 °C in a 'Mr frosty' (Nalgene, UK), filled with Isopropanol. Cells were stored at -80 °C for 48 h before being transferred to liquid nitrogen for long term-storage. Cells that would be required within 6 months of freeze down were maintained at -80 °C. The use of FDM and slow programmable freezing facilitated by Isopropanol, which reduces temperature in a controlled manner (1 °C/min), protects cells against intracellular and extracellular ice formation and dehydration which occurs during the freeze down stage of cryopreservation.

For the recovery of frozen aliquots, cryovials were removed from -80 °C or liquid nitrogen and immediately placed in a water bath set at 37 °C until completely thawed. Once thawed vials were removed from the water bath and cleaned with 70% Industrial methylated spirit (IMS). The aliquots of cell suspension were then transferred into 5ml bijous to which an equal volume of culture media was added in a drop-wise fashion over 1 min to prevent osmotic shock and maximise the number of viable cells

recovered. The cell suspension was then added to an additional 5 ml of culture media and transferred to a 15 ml conical centrifuge tube. Cells were centrifuged for 5 min at 800rpm to pellet the recovered cells. The supernatant was carefully removed and the cell pellet re-suspended in normal growth before transfer to a T25 culture flasks. Once cells reached ~70% confluence in T25, flasks cells were transferred and maintained in T75 tissue culture flasks.

2.2 The Experimental Model

2.2.1 Desmoglein 3 Gain-of-function

Stable overexpression

In order to examine the signalling role of Dsg3, the unique retroviral construct of full length human Dsg3 (hDsg3.myc) was transduced into A431 cells (Tsang, et al., 2010). A431 cells made a suitable model in this study as they exhibit a low level of endogenous Dsg3 expression. The transduction of Dsg3 in A431 cells resulted in a stable increase in Dsg3 protein expression (at least 2-fold, compared to vector control). The construct for full length human Dsg3 with a *myc*Tag epitope located at the C-terminus (hDsg3.myc) was successfully cloned and constructed into the pBABE.puro retroviral vector (pBABE-hDsg3.myc) by Dr Hong Wan (Wan, et al., 2007). The empty vector control (pBABE-puro), as well as the *myc*Tag control (pBABE-*myc*Tag), was used alongside the hDsg3.myc construct. The constructs were packaged into retroviruses by Dr Ahmed to generate condition media for infection of target cells, A431 and SqCC/Y1.

For infection, A431 parental cells were seeded to achieve 30% confluence following overnight culture. The following day growth media was replaced with the respective retroviral condition media (filtered through 0.45 µm) supplemented with polybrene (8 µg/ml). Polybrene increases the efficiency of transduction by neutralising the charge repulsion between virions and sialic acid of the cell membrane. To further increase infection efficiency the cell cultures with condition media were centrifuged at 360rpm at 32 °C for 1hour and then placed back in an incubator. After 48 h of infection, condition media was replaced with normal growth media and allowed to recover for 2-3 days before drug selection for successfully transduced cells. For selection, cells were cultured in normal growth media supplemented with puromycin (2 µg/ml) for a period of 2 weeks. During this time period a majority of cells died leaving behind a polyclonal population of cells with positive expression of hDsg3.myc.(A431-D3) After selection

cells were maintained in normal growth media, a recovery period of a week was adhered to prior to experimentation and cryopreservation.

Generation of single cell clones

Limiting dilution followed by clonal expansion was carried out with A431-D3 polyclonal cells (Courtesy of Dr M Tsang). In brief, A431-D3 cells suspended in normal growth media were subjected to a series of increasing dilutions to achieve a cell concentration of ~10 cells/ml. Aliquots of 100µl of the cell suspension were then seeded into a 96-well plate. One week later each well was examined and those identified as having a single colony were allowed to propagate for several weeks. These clones were trypsinised and transferred to T25 culture flasks for further clonal expansion. Aliquots from each clone were then analysed by both Western blot (courtesy of Dr M Tsang) and real time qPCR (courtesy of Dr Tech) to characterise the level of Dsg3 expression on both a translational and transcriptional level (data not shown). High and low expression clones were then selected and used in this study alongside the vector control (Vect Ct) and polyclonal A431-D3 cells. All monoclonal population derived from A431-D3 cell are denoted by the affix 'C#', for example, the high expression clone, A431-C7 and low expression clone, A431-C11.

Transient overexpression.

In preparation, A431-parental cells (2×10^5) were seeded into each well of a 6-well plate 24 hours before transfection. The following method is for the transfection of one well in a 6-well plate. In a 1.5 ml polystyrene microcentrifuge tube 100µl of Opti-MEM (Gibco) and 1 µg of either hDsg3.myc or Vect Ct cDNA was added, the solution was mixed gently by pipetting. Then, 6 µl of Eugene HD (Promega) was added directly into the Opti-MEM:cDNA solution with care taken not to touch the tube wall. After gentle mixing the transfection solution was incubated for 15 min at room temperature (RT). In the meantime, the media of the prepared A431 cells was replaced with 2.5 ml of fresh growth media. Following the 15 min incubation period the transfection solution was added to the cells in a drop-wise fashion. Transfection was carried out for 24 h after which, cells were trypsinised and re-seeded at the desired concentration for the assay to be carried out 48 h after transfection.

Table 2.1 siRNA sequences, target genes, manufacturer and working concentrations.

siRNA	Gene target	Manufacturer	[Working]
AAATGCCACAGATGCAGAT GA*	Human Dsg3 (Accession: NM_001944.1): 620- 640	Designed by Dr Hong Wan and synthesized by Dharmacon	50 nM
CAUAGCAUUAUCUUUGAG C	Ezrin	Dharmacon	50 nM
AAAUUCGUCAAUGCGCUG C	Moesin	Dharmacon	50 nM
AACGATGATACATGACACG AG	Scrambled control	Dharmacon	50 nM

*Scrambled sequence was used as a scrambled control siRNA

2.2.2 Desmoglein 3 Loss of function

Transient siRNA transfection for Dsg3 silencing.

The protocol of siRNA transfection and a significant reduction of Dsg3 are shown previously (Mannan, et al., 2011). In brief, A431-parental cells (2×10^5) were seeded in each well of a 6-well plate in normal growth media 24 h prior to transfection. The following method is for a transfection of one well in a 6-well plate. For siRNA transfection, two solutions (A and B) were prepared and then combined to give rise to a siRNA transfection solution of 200 μ l. For solution A, scrambled control or target specific siRNA (see Table 2.1) was added to Opti-MEM to achieve a siRNA concentration of 0.25 μ M in 185 μ l of Opti-MEM. For solution B, 5 μ l of oligofectamine (Invitrogen) and 10 μ l of Opti-MEM were combined. Both solution A and B were mixed gently by pipetting and incubated at RT for 15 min. Solution A and B were then combined, mixed by gentle pipetting and incubated for a further 20 min at RT. In the meantime, the prepared cells were washed three times with Opti-MEM before the addition of 800 μ l of Opti-MEM. Once the incubation of the transfection solution was complete it was added to the culture in a drop-wise fashion. Transfection was allowed to occur for 4 hours before the addition of 500 μ l of Opti-MEM supplemented with 30% FCS to achieve a final concentration of 10%FCS as a routine. The following day cells were trypsinised and re-seeded at the desired density for assay 48 h after transfection. All transfections had a final siRNA concentration of 50 nM.

2.3 Molecular Biology Methods

2.3.1 Western blot

Before cell lysis, media were swiftly removed before placing the culture plate on ice. Total cell lysates were extracted using 2x sample buffer (see below for volumes) and collected with the aid of a cell scraper (BD, UK). Lysates were then transferred to pre-labelled 1.5 ml microcentrifuge tubes and boiled for 5 min at 95-100 °C to reduce the viscosity of the sample, before being placed on ice. Samples for SDS-PAGE at a later date were stored at -80°C at this stage.

In all Western blots in this study an equal protein concentration of samples was loaded into gels. Protein concentration was determined using the *DCTM* protein assay kit (Bio-Rad) which is a colorimetric assay compatible with SDS containing buffers. Briefly, 5 µl of each test sample was loaded into a 96-well plate alongside a set of bovine serum albumin standards (0, 0.25, 0.5, 0.75, 1.0, 1.52, 2.5 and 5 µg/ml) to which 25 µl of Reagent A (solution A + solution D_c, 50:1) followed by 200µl of Reagent B were added. The blue colour was allowed to develop for 15 min at RT followed by the measurement of sample absorbance using a plate reader. Absorbance of each sample and standard was obtained at a wavelength of 650 nm. The protein concentration of test samples was extrapolated from the standard curve generated from the BSA standards. Once protein concentration was obtained a portion of each sample was combined with an equal volume of sample buffer containing bromophenol blue dye to assist gel loading. All samples were boiled again for 2 min at 95-100 °C prior to centrifugation at 13,000xg for 1 min. An equal amount of total protein was loaded into each lane of the appropriate gel (see below) ranging from 5-40 µg depending on target protein abundance and volume of lysate available. At least one lane in each gel was reserved for a molecular weight marker (full range rainbow marker, Invitrogen).

All samples were resolved by SDS-PAGE using the NuPAGE®SDS-PAGE gel system (Invitrogen), followed by electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membrane carried out at 30 V for 90 min at 4 °C. The nitrocellulose membranes were then subjected to blocking of non-specific binding sites by incubation with blocking buffer for 30 min at RT or overnight at 4 °C. Membranes were then incubated with primary antibodies diluted in blocking buffer (see Table 2.3 for antibody concentrations) for 1 h at RT or overnight at 4 °C. After three washes (3x5

min) in TTBS the membranes were incubated with secondary antibodies conjugated with HRP diluted in blocking buffer (1:1000) for 1 h at RT. Finally, after 3x5 min washes in TTBS the membranes were subjected to the Enhanced Chemiluminescence (ECL) Plus *Western Blotting* detection system (Amersham) or Femto Chemiluminescent Substrate (Pierce) to detect target proteins according to the manufacturers specifications, and recorded using hypersensitive ECL film (GE Healthcare Bio-Sciences Corp).

2x sample buffer

- 0.14 M Tris –HCL
- 4.5% SDS
- 22.5% Glycerol

In H₂O

Running buffer (Invitrogen)

- NuPAGE® MES SDS running buffer (20x): For Bis tris gels
- NuPAGE® Tris-Acetate SDS running buffer(20x): for Tris Acetate gels

1x Transfer buffer

- 25 mM Tris Base
- 192 mM Glycine
- 20% Methanol

1x TTBS

- 0.1 M Tris buffer (pH 7.5)
- 0.1% Tween 20
- 154 mM Sodium Chloride

Blocking Buffer

- 5% non-fat dry milk in TTBS

2x Sample buffer (plus Bromophenol blue)

- 0.12 M Tris -HCL
- 3.8% SDS
- 18.4% Glycerol
- 0.009% Bromophenol Blue
- 9.1% Mercaptoethanol

Gels used (NuPAGE® Invitrogen)

- General use= 1mm, 4-12% and 10% BisTris precast gels 10 - 12 wells
- pERM=1mm, 3-8% Tris Acetate precast gels 10 well

Cell lysis volumes

- 12 well tissue culture plate 50 ul
- 6 well tissue culture plate=75 ul
- 10cm tissue culture plate=300 ul

Western blot analysis.

The films of Western blots with different exposure were scanned using a flatbed desktop scanner at a minimum of 600 dpi and the band density of blots was determined using ImageJ. As shown in Figure 2.1A, a set of blot bands for a target protein were selected and analysed together Figure 2.1A shows bands densities for Dsg3 expression in A431-Vect Ct, -D3, -C7 and -C11 cells, respectively. This selection was then identified as the 1st lane (Analyze>Gels>Select 1st lane) followed by 'Plot

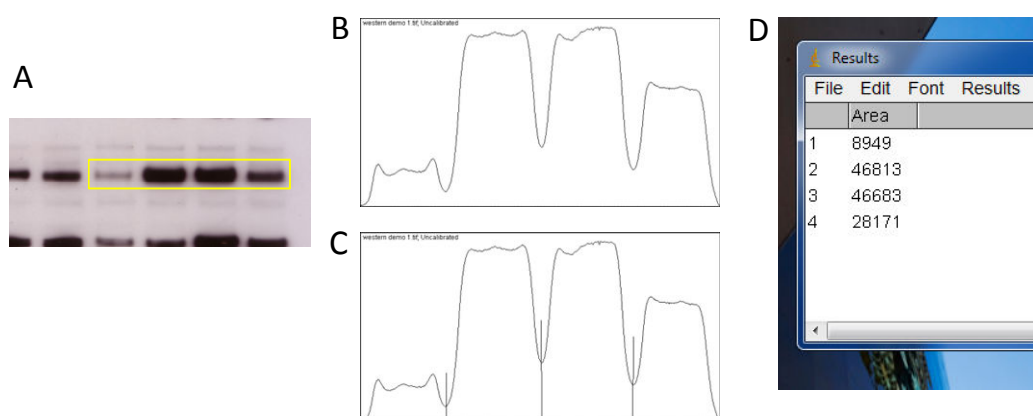


Figure 2.1. Western blot analysis using ImageJ. (A) bands selected for analysis. **(B)** Band density plot with ImageJ. **(C)** Blot peaks separated by lines to allow quantification of each peak individually. **(D)** Quantification of individual peaks.

lanes' (Analyze>Gels>Plot lanes) to generate a 'Western demo plot' (Figure 2.1B). The lanes were then divided using the line tool to allow for the area of each peak to be determined separately (Figure 2.1C). Using the 'Wand (tracing) tool' each peak was selected to generate a band area (Figure 2.1D). The peak values were then exported into an Excel spreadsheet where all values were normalisation against a loading control (GAPDH, β actin or α Tubulin). Once normalised band density were expressed relative to the vector control or scrambled control density set at 1 arbitrary unit. Data presented as mean fold change \pm SD with respect to the control.

2.3.2 Experiment with inhibitors

For experiments with pharmacological inhibitors in conjunction with calcium switch, cells were seeded at sub-confluence and allowed to adhere for 3-4 h. Once adhered cells were subjected to serum starvation by replacing normal growth media with serum-free DMEM and incubated overnight. The following day the serum-free DMEM was replaced with calcium-free DMEM without serum for <1 h under close inspection to ensure cells were rounded but did not detach. Media was replaced with either calcium-free DMEM supplemented with 10% decalcified serum or normal growth media, in the presence or absence of inhibitors. DMSO was used as vehicle control in the absence of inhibitor. Cells were incubated with or without the inhibitors for 5 hours before cells were lysed for examination by Western blot.

Calcium free DMEM w/o Serum

- 3 mM EGTA in DMEM

Calcium free DMEM with calcium free Serum

- 3 mM EGTA
- 10% decalcified FCS

Table 2.2 Pharmacological inhibitors, their targets, working concentrations and incubation time periods.

Inhibitor	Target	Concentration	Incubation time	Manufacturer	Reference
BIM	PKC	5 μ M	0.5, 1 & 5 h	Cell signalling	(Rigot, et al., 1998)
		10 μ M	5 h		
		0.5-25 μ M	4 h		
Rö 31-7549	PKC	2 μ M	5 h	Calbiochem	(Moon, et al., 2009)
		10 μ M	5 h		
Gö 6976	PKC	1 μ M	5 h	Calbiochem	(Tomic, et al., 2011)
SP600125	JNK	10 μ m	4 h	Sant Cruz	(Moon, et al., 2009)
SB 203580	P38	20 μ M	5 h	Calbiochem	(Wang and Ron, 1996)
NSC23766	Rac1	30 μ M	5 h		(Gao, et al., 2004)
C3	RhoA	0.1 μ g/ml	5 h	Cytoskeleton Inc	(Waschke, et al., 2006)
Y-27652	ROCK	10 μ M	4 h	Ascent	(Sahai and Marshall, 2002)
PP2	Src	10 μ M	5 h	Calbiochem	(Nam, et al., 2002)

Calcium-free serum was prepared as follow (courtesy of Dr Tsang). Briefly, the chelating resin was mixed into dH₂O (250 ml of water/5 g of resin) for 1 hour at RT, pH was then adjusted to 7.4. After 3 h equilibration the resin:H₂O mix was filtered through Whatman No1 filter paper. The equilibrated resin was then mixed with FCS overnight at 4°C (5 g of Chelex resin/50ml of FCS). The following day resin was removed via centrifugation at 1500xg for 10 min. The resulting serum was then sterilised by passing through a 0.22 μ m filter, 50ml aliquots were stored at -20°C until required.

For other experiments with the inhibitors, cells were seeded and serum starved as described above. The following day cells were treated with various inhibitors that were diluted in normal growth media (see Table 2.2 for concentrations and incubation time periods). Finally, cells were lysed and analysed by Western blot.

2.3.3 Co-immunoprecipitation assay

Cells were seeded at sub-confluent density in T75 flasks and grown overnight. Cells were then washed with ice-cold PBS and lysed on ice for 10 min with ice cold 1x RIPA buffer (Upstate) containing a protease inhibitor cocktail. The lysates were collected with aid of a cell scraper and transferred to microcentrifuge tubes prior to centrifugation at 13,000xg for 5 min to eliminate cell debris. The supernatant were then carefully transferred to a fresh pre-labelled microcentrifuge tube and protein concentration for each sample was determined by the DC™ protein assay kit (Bio-Rad) as described in the Western blot protocol. For immunoprecipitation, 0.5-1mg of protein was incubated with 3-5 µg of primary antibody at 4°C overnight on a rotation wheel. The following day 1.7x10⁵ Protein G magnetic Dynabeads® (Novex, Invitrogen) were added to the Lysate:antibody solution and incubated for a further 1 hr at 4°C on a rotation wheel. The bead-bound immunoprecipitates were washed 4x in RIPA buffer and 1x in 1xTTBS. The resulting immunoprecipitates were re-suspended in 11 µl of 2x sample buffer and boiled for 2 min at 95-100°C. Finally, the proteins of the immunoprecipitates were resolved by 4-12% or 3-8% SDS-PAGE followed by procedures described in the Western blot protocol.

1xRIPA Buffer

- 1x RIPA solution (Upstate Biotech)
- 1 mM Sodium Orthovanadate
- 15 mM sodium fluoride
- 1 mM PMSF

Just before use protein inhibitor cocktail was added (1:1000)

1xTTBS

- 0.1 M Tris buffer (pH 7.5)
- 0.1% Tween 20
- 154 mM Sodium Chloride

2x Sample buffer (plus Bromophenol blue)

- 0.12 M Tris -HCL
- 3.8% SDS
- 18.4% Glycerol
- 0.009% Bromophenol Blue
- 9.1% Mercaptoethanol

Volume of RIPA buffer

- 10cm tissue culture plate=300 µl
- T75 tissue culture flask = 500 µl

2.3.4 Cell fractionation

Cells were seeded to achieve sub-confluence following overnight incubation. The following day cells were lysed using Triton X-100 buffer on ice for 10 min to extract Triton soluble proteins. The Triton insoluble proteins were precipitated out of solution by centrifugation at 14,000xg for 30 min at 4°C. The supernatant (Triton soluble

protein) was carefully transferred to a fresh pre-labelled microcentrifuge tube and kept on ice. The remaining pellet (Triton insoluble protein) was solubilised in 20 µl of 2x sample buffer. Protein concentration of each sample was determined as described in the Western blot protocol. Consistent amounts of Triton soluble and Triton insoluble protein were loaded and resolved by 4-12% or 3-8% SDS-PAGE followed by procedures described in the Western blot protocol.

Triton X-100 Buffer

- 10 mM Tris-HCl
- 1% Triton X-100
- 145 mM NaCl
- 2 mM EGTA
- 5 mM EDTA
- 1 mM PMSF
- 10 mM Sodium orthovanadate
- 10 mM Sodium fluoride

Just before use Protease inhibitor cocktail was added (1:100)

Volume of Triton X-100 buffer

- 10 cm tissue culture plate=300 µl
- T75 tissue culture flask = 500 µl

2x Sample buffer (plus Bromophenol blue)

- 0.12 M Tris -HCL
- 3.8% SDS
- 18.4% Glycerol
- 0.009% Bromophenol Blue
- 9.1% Mercaptoethanol

2.3.5 Luciferase assay

In preparation cells were subjected to transient co-transfection with AP1 luciferase reporter and hDsg3.*myc* constructs 2 days before the luciferase assay. Briefly, cells were seeded at ~30% confluence in a 6-well plate and allowed to propagate overnight in normal growth media. The following method is for a transfection of one well in a 6-well plate. In a 1.5 ml polystyrene microcentrifuge tube 100 µl of Opti-MEM (Gibco) was added followed by 2 µg of cDNA directly into the media with care taken not to touch the tube walls. The solution was mixed by gentle pipetting. The 2µg of cDNA added was a mixture of 0-1 µg pBABE-hDsg3.*myc* together with 1 µg of wild type pGL-AP-1 luciferase reporter or its mutant or empty pGL-Vect Control. The amount of pBABE-hDsg3.*myc* cDNA when less than 1 µg was substituted using the pBABE-GFP cDNA in order to make the total concentration of cDNA transfected consistent at 2 µg for all co-transfections. Next, 6 µl of Eugene HD (Promega) was added into the Opti-MEM:cDNA solution to complete the transfection solution which was mixed gently for 15 s followed by incubation at RT for 15 min. In the meantime, the media of the prepared cells were changed with 2.5 ml of fresh growth media. Following the 15 min incubation the transfection solution was added to the culture in a drop-wise fashion.

Transfection was allowed to occur for 24 h. Cells were then harvested and re-seeded into 12-well plates to achieve sub-confluence and incubated for a further 24 h.

For experiments without inhibitors, 48 h after transfection the luciferase assay was performed using the Progmega Luciferase Assay System. Here, cells were lysed using 70 µl/well of 1xReporter lysis buffer (Promega, E397A) for 5min on ice and collected with the aid of a cell scraper. Lysates were then transferred to fresh microcentrifuged tubes and frozen immediately in dry Ice for 30 min and then thawed. Samples were vortexed for 15 s followed by centrifugation for 15secs at room temperature. Five µl of each sample was used to determine protein concentration as described previously, and 10-15 µl aliquots of the remaining sample were assayed in duplicate or triplicate, depending on the amount of lysates available, in a white flat bottom 96-well plates. Once loaded, the plate was taken near the plate reader which was set to detect luminescence. Then, 100 µl of luciferase assay reagent (Promega, E151A+E152A) was added to each well and the plate reading initiated immediately. Each reading was normalised against protein concentration and volume of lysate in order to determine Luciferase activity per µg of protein. An average of duplicate or triplicate readings was then determined in an Excel spreadsheet and presented as Mean fold change in Luciferase activity/µg of protein \pm SD.

For experiments with inhibitors, the transfected cells were re-seeded into a 12-well plate 24 h after co-transfection and allowed to adhere for 3-4 h before serum starvation overnight. The following day cells were treated with inhibitors diluted in normal growth media (the concentrations and incubation time periods of the inhibitors are illustrated in Table 2.2). Once complete, the Luciferase assay was performed as described above.

2.3.6 Real-time absolute quantitative RT-PCR

Standard Curve

In preparation, 50 µl PCR reactions were run in 96-well plates to amplify a large quantity of PCR product for Dsg3. Each reaction contained the following:

- | | |
|-----------------------------|-------|
| • 2x SYBR Green 1 Mastermix | 25 µl |
| • dH ₂ O | 15 µl |
| • Template | 5 µl |
| • 5uM Dsg3 F/R primer | 7 µl |

At this stage the template was from a cocktail cDNA. The Dsg3 primers used were as follows, *Forward* 'CCGAATCTCTGGAGTGGGAA', *reverse* 'GCCCCAAGGACTAGATGTAGA' which yield a 164 bp product. The resulting PCR product was purified in MinElute PCR purification spin columns according to the manufacturer's specifications to yield a 20 µl DNA elute in dH₂O. The DNA concentration was determined using Nanodrop and a Dsg3 DNA stock with a concentration of 10¹¹ copies/2µl was achieved using the following equation:

$$\left(\frac{6.02 \times 10^{14}}{(\text{basepair})/(660)} \times [\text{DNA}] \times 2 \times \text{vol} \right) - \text{vol} = \text{vol. to generate } 10^{11}/2\mu\text{l DNA stock}$$

From the 10¹¹ stock, a series of dilution was made to generate the following standards of 10⁹, 10⁷, 10⁶, 10⁵, 10⁴, 10³ and 10² copies per 2µl. The resulting standards were stored at -20°C until required. To set up the standard curve, a 96-well plate was used and each well contained the following:

- 2x SYBR Free master mix 5 µl
- 5µM Dsg3 F/R primers 1 µl
- dH₂O 3 µl
- Template 2 µl

Here, the template used was the Dsg3 standards. Once loaded the plate was sealed with a Roche sealing film and centrifuged for 30 s at ~3000 rpm. The qPCR was carried out on the LightCycler 480 (Roche) using the following protocol:

- Denaturation (hot start) 95°C 5 min
- Amplification (45 Cycles)
 - Melting 95°C 10 s
 - Annealing 60°C 6 s
 - Extension 72°C 6 s
 - Acquisition 76°C 1 s
- Melting Analysis
 - Melting 95°C 30 s
 - Cooling/annealing 65°C 30 s
 - Gradual heating + acquisition 65-99°C
 - Cooling/termination 40°C 5 s

The standard curve generated is stored in the LightCycler 480 software for application in subsequent experiments.

Real-time absolute quantitative RT-PCR

Here, A431 cells were seeded in a 6-well plate to achieve sub-confluence followed by culture overnight. The mRNA was purified from the cells using Dynabeads®mRNA Direct kit (Invitrogen) according to the manufacturer's specification. Reverse transcription reactions were carried out in 0.2 ml PCR tubes, each reaction contained the following using Transcriptor cDNA Synthesis kit (Roche):

- 5x buffer 4 µl
- dNTP 2 µl
- Random+OligoDT primers 0.8 µl
- RNasin 0.4 µl
- Reverse Transcriptase 0.4 µl
- mRNA 13 µl

Reverse Transcription reactions were carried out using a thermocycler block with the following protocol 42°C 30 min, 85°C 5 min and 4°C 5 min. The resulting cDNA was diluted in 40 µl of dH₂O and stored at -20°C until required.

For qPCR of the cDNA products of A431 cells, reactions were set up and ran as described above for the generation of the standard curve. The absolute copy number was determined by normalising against reference genes and compared to the Dsg3 standard curve.

2.3.7 Mass spectrometry

The following method provided by Dr Isa Cruz

SDS-PAGE

Samples were mixed with 1x SDS loading buffer and, after denatured for 10 min at 95°C, they were loaded onto a precast gel, Mini-Protean TGX Precast Gels, any kD, 10-well comb, 30 µL/well (Bio-Rad). Electrophoresis was carried out on a Mini-Protean Tetra Cell System (Bio-Rad) at 150 V, using 1x Tris-Glycine-SDS running buffer, until the dye front had reached the bottom of the gel. Proteins were visualised with silver stain (Pierce Silver Stain Kit, Thermo Scientific).

Native-PAGE

1x native loading buffer was added to each sample and the mixtures were loaded into the wells of a 10% polyacrylamide native gel. Proteins were separated in the absence of SDS by native gel electrophoresis at 150 V and visualised with silver stain (Pierce Silver Stain Kit, Thermo Scientific).

Protein Band Excision and In-gel Trypsin Digestion

Bands of interest were excised and cut in 1 – 2 mm³ gel pieces, which were placed into 0.6 mL siliconized tubes. Gel pieces were rinsed twice with 190 µL of wash solution (50% methanol + 5% acetic acid) at room temperature; the first wash was for 3 h and the second overnight. Wash solution was removed and gel pieces were dehydrated in 190 µL of acetonitrile at room temperature for 5 min. After that, acetonitrile was removed from the samples and they were dried in a vacuum centrifuge for 3 min. Sample reduction was then performed with 30 µL of 10 mM DTT at room temperature for 30 min, followed by alkylation with the same volume of 100 mM iodoacetamide at room temperature for another 30 min. Iodoacetamide solution was removed from samples and they were dehydrated once again in 190 µL of acetonitrile at room temperature for 5 min and dried in a vacuum centrifuge for 3 min. Gel pieces were rehydrated in 190 µL of 100 mM ammonium bicarbonate at room temperature for 10 min. After removing the previous solution, gel pieces were dehydrated in acetonitrile and dried in a vacuum centrifuge another time.

Finally, samples were rehydrated on ice, for 10 min, with 20 µL of trypsin solution (20 ng/µL sequencing grade modified porcine trypsin (Promega) in 50 mM ice-cold ammonium bicarbonate pH 8.0) with occasional vortex mixing. After rehydration, samples were spun down for 30 sec and excess trypsin solution was removed. An aliquot of 10 µL of 50 mM ammonium bicarbonate solution was added to the gel pieces to prevent dehydration and proteins were digested overnight at 37°C.

Peptide Extraction from Gel Pieces

To begin with, 30 µL of 50 mM ammonium bicarbonate solution were added to the digests and, after a 10 min incubation period, supernatant was collected and transferred to a new tube. Peptides were firstly extracted from gel pieces with 30 µL of extraction buffer I (50% acetonitrile + 5% formic acid), incubation for 10 min and supernatant collection. A second extraction was performed with 30 µL of extraction buffer II (85% acetonitrile + 5% formic acid) for 10 min, followed by a 30 sec centrifugation and supernatant collection. After extraction, the extracts volume was reduced to <5 µL by evaporation in a vacuum centrifuge. Finally, the dried extracts were redissolved in 0.1% formic acid, sonicated for 10 min, centrifuged for 5 min and transferred to mass spec vials.

LC-MS/MS Analysis

LC-MS/MS analysis was performed on a Waters CapLC system coupled to the front end of a Waters Micromass Q-ToF Premier. Depending on the intensity of the spot on the 2-D gel, 1-5 μ L were injected per sample. A 63 min gradient LC method was followed: t = 0.1 min, 5% B; t = 3 min, 5% B; t = 40 min, 28% B; t = 49 min, 80% B; t = 52 min, 80% B; t = 53 min, 5% B and t = 63 min, 5% B (A, 5% ACN + 0.1% formic acid (FA); B, 95% ACN + 0.1% FA). Initial flow from the CapLC was at 6 μ L/min, which was split prior to the column so the flow through the column to the mass spectrometer was only 200 nL/min. Peptides eluted off the column were directly sprayed into the mass spectrometer for analysis. The acquisition and processing software used was Waters MassLynx Version 4.1. MS was monitored over a m/z range of 400-1700 Da and MS/MS was monitored over a m/z range of 50-1700 Da. Capillary was at 1.8 kV, cone voltage at 35 V and collision energy at 35 eV.

MS/MS data were used to perform database searching using an online search engine, Mascot, choosing SwissProt databases to look for human proteins (taxonomy - *Homo sapiens*). Searches were performed without restriction of protein molecular mass or pI, but with variable modifications such as carbamidomethylation of cysteines and oxidation of methionine residues. One trypsin miscleavage was allowed. Peptide and fragment mass tolerances were set to ± 0.1 Da.

2.4 Cellular Biological Methods

2.4.1 Immunostaining

Cells were seeded on coverslips to achieve sub-confluence followed by culture overnight. Twenty-four hours later, cells were washed twice with PBS and fixed with either ice-cold methanol:acetone (1:1) or 3.8% formaldehyde (F-actin) or 4% Para-formaldehyde (pERM), for 10 min. Cells fixed with formaldehyde or para-formaldehyde were permeabilised with 0.1% Triton X-100 for 10 min (F-actin) or 1 min (pERM). Cells were washed 3x with PBS before immunostaining or stored in PBS at 4°C for later use.

For fluorescent staining, coverslips were blocked for 30 min with blocking buffer followed by incubation for 1hr at RT with target specific antibody diluted in blocking buffer (see Table 2.3). Cells were then washed 3x with IMF washing buffer followed by incubation for 1hr at RT with Alexa Flour 488 and 546 conjugated secondary antibodies

diluted in blocking buffer (1:100). Cells were then washed 2x with washing buffer followed by a 10 min incubation with DAPI (1:1000) for nuclear counter-stain before a final wash. Coverslips were then mounted on slides using ProLong®Gold anti-fade liquid mount (Invitrogen) and sealed with clear nail varnish. Coverslips were imaged using either Leica DM5000 epi-fluorescence microscope, Zeiss 710 LSM confocal microscope, Meta 510 LSM confocal microscopes or MM Leica epi-fluorescence microscope.

Blocking buffer

- 10% goat serum in IMF washing buffer

IMF Washing Buffer

- 0.2% Tween 20 in PBS

Fixatives

- General staining: Methanol:Acetone (1:1)
- F-actin and pERM staining: 4% Para-formaldehyde or 3.8% formaldehyde

2.4.2 Immunofluorescence image analysis and Acquisition

Images were acquire using the following microscopes:

MM-Leica DM4000 microscope fitted with a QIClick Qimaging 12 bit monochrome 1.4 megapixel camera which contains a Sony IC285 CCD chip for image acquisition. Image acquisition software used was Metamorph v7.7.10. Filter cubes used: DAPI Band Pass 400nm +/- 20nm, FITC - Band Pass 490nm +/- 20nm and Rhodamine - Band Pass 560nm +/-20nm. Images were acquired using a 20x, 40x oil or 63x oil objective. Acquired images were exported as Tiff files

Leica epi-flouresence DM5000 microscope, fitted with a Leica DFC350Fx 12 bit monochrome 1.4 mega pixel camera with a Sony IC285 chip for image acquisition. Image acquisition software used was Metamorph v7.7.10. Filter cubes used: DAPI Band Pass 400nm +/- 20nm, FITC - Band Pass 490nm +/- 20nm and Rhodamine - Band Pass 560nm +/-20nm. Images were acquired using a 20x, 40x oil or 63x oil objective. Acquired images were exported as Tiff files.

Zeiss LSM710 Meta confocal microscope fitted with a 2 x Photomultiplier tubest (PMTs) 1 Quasar spectral detector 10nm grating step size detection system. Image acquisition software used was Zen 2012 Laser lines used: 458nm, 477nm, 488nm,

514nm Argon laser lines Images were acquired using a 20x, 40x oil, or 63x oil objective lens. Acquired images were exported as LSM files.

Zeiss LSM710 meta microscope fitted with a 2 x Photomultiplier tube (PMTs) 1 Quasar spectral detector 10nm grating step size detection system. Image acquisition software used was Zen 2012. Laser lines used: 458nm, 477nm, 488nm, 514nm Argon laser lines. Images were acquired using a 20x, 40x oil, or 63x oil objective lens. Acquired images were exported as LSM files.

Nuclear number: Using ImageJ software, channels for each image were split and the DAPI channel selected for nuclear number determination. A 'Threshold' was set to select all nuclei, followed by conversion to a binary format. In some cases during thresholding, some background was detected, and this background was removed using the processing tools 'erode' and 'despeckle'. The 'watershed' tool was then applied to split nuclei that are touching. The nuclear number was then determined using the 'analysis particles' tool.

Cell area: Using ImageJ, channels of each image were split, with the red and green channels analysed individually. Once a channel was selected, a 'threshold' was set to select the area covered by the cells, the same threshold was maintained the analysis of all images for that channel for each experiment. Cell area was measured (analyse>measure). Total cell area for each field was then divided by the nuclear number to generate an average cell area/field. At least three randomly selected fields were analysed from each coverslip and presented as mean \pm SD in figures.

Colocalisation analysis: Using WCIF ImageJ, channels for each image were split, only the red and green channels were used for analysis. Each channel was converted to a 8-bit format for the determination of percentage colocalisation (Plug-in>colocalisation finder). The percentage colocalisation generated was then divided by the number of cells in each field. At least three randomly selected fields from each coverslip were analysed. Data presented as the mean percentage colocalisation/field \pm SD in figures.

Various colocalisation co-efficient were also calculated using the WCIF plug-in 'Intensity correlation analysis' (Plugins > colocalisation analysis > Intensity correlation analysis). In the same way, channels were split and the red and green channel were

analysed. Using the 'Intensity correlation analysis' plug-in, Pearson's correlation Coefficient, Mander's Overlap Coefficient and Intensity Correlation Quotient were calculated. At least three randomly selected fields from each coverslip were analysed and data presented as average Coefficient value \pm SD.

Integrated density: Using ImageJ, channels for each image were split and the red and green channels were analysed. Thresholds for each channel were set individually to select positive fluorescence and exclude the background before the integrated density was measured (Analyse>measure). The same threshold was applied to all images across different samples for each channel. All integrated density were normalised by the cell count per field determined from the nuclear count (see above) to yield integrated density per cell. A minimum of 3 field per cover slip were analysed in this way to yield an average integrated density per cell line. In figures Integrated density per cell is referred to as 'integrated density'. Integrated density as a function of target protein expression is expressed as either average fold change relative to the vector control or scrambled ct levels set at 1 arbitrary unit (data displayed as Mean fold change \pm SD with respect to the controls); or relative integrated density where the average integrated density per cell line is presented (data displayed as integrated density / cell \pm SD).

Staining Profiles

For staining profiles of Dsg3, Ezrin, Radixin, Moesin, images were analysed using ImageJ. Here a bisecting line of defined width (20) was used, as illustrated in Figure 2.2A. Once in place the profile of fluorescent staining along the line was plot (Analyze>Plot profile) to generate a histogram similar to that in Figure 2.2B. The plot values as shown in Figure 2.2C were obtained, copied and exported to an Excel spreadsheet where the profiles of >70 cells were collated. Data are presented as the mean Grey value \pm SD.

For the analysis of nuclear staining profiles of cJun, the same approach was used as described above, except the bisecting line was reduced in width (10) and limited to the nucleus only. Data are presented as the mean Grey value \pm SD

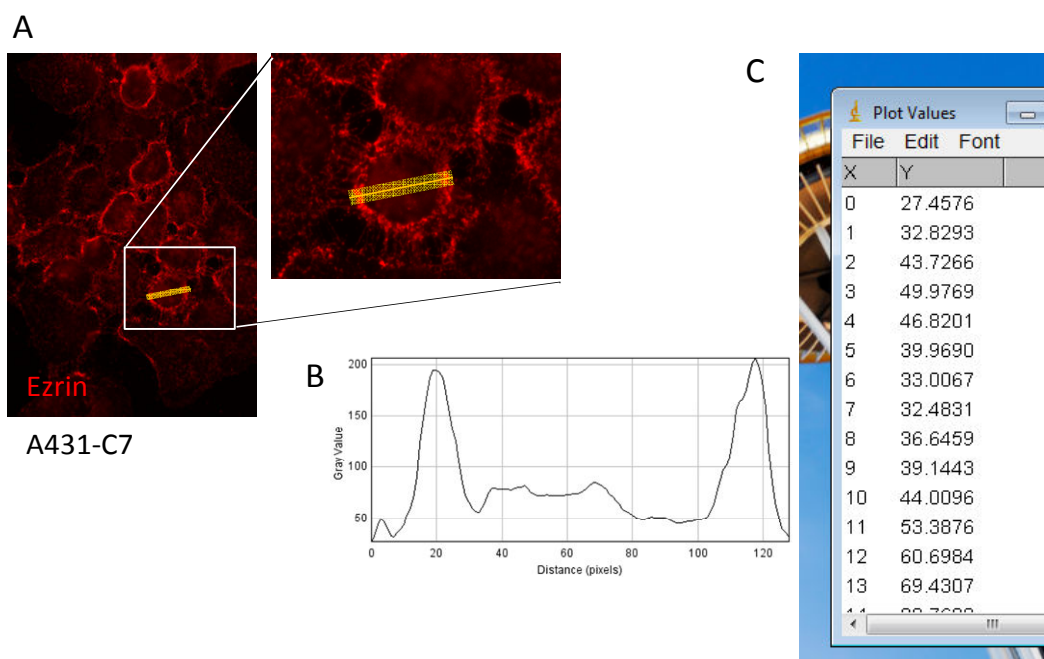


Figure 2.2. Analysis of individual cell staining profile. (A) A selected cell bisected with a line with 20 (arbitrary unit) in width. **(B)** Plot of immunofluorescence profile of the bisecting line in (A). **(C)** The actual values of the plot then can be exported to Excel for further analysis.

2.4.4 Analysis of Triton soluble and insoluble protein

Cells were seeded on coverslips to achieve sub-confluence following overnight incubation. Twenty-four h later cells were incubated with 0.1% Triton buffer or PBS as a control, for 6 min. Cells were then washed 3x with ice cold PBS and fixed with 3.8% PFA on ice. Cells were finally stained as described in Part 2.4.1, and images were acquired and analysed using ImageJ to determine integrated density.

2.4.5 FRET (Fluorescent Resonance Energy Transfer) by acceptor photo-bleaching

FRET occurs on the principle that, between a FRET pair (donor and acceptor), the excitation energy of the donor is transferred to the acceptor without the emission of a photon in close proximity. Therefore positive FRET occurs when the excitation wavelength of the donor results in the fluorescence of the acceptor molecule alone. Assessment of FRET by acceptor photo-bleaching is one of various methods of detecting FRET. Here, photo-bleaching of the acceptor molecule inhibits its ability to be excited, resulting in the un-quenching of the donor. Experimentally this will be observed as an increase in donor fluorescence. Cells were seeded on coverslips to achieve sub-confluence following overnight culture. Cells were fixed (ice cold Methanol:Acetone) and labelled with primary antibodies specific to Dsg3 (5H10) and Ezrin as described for immunofluorescence. The secondary antibodies used for FRET

analysis were mouse and rabbit IgGs conjugated with Alexa Fluor 488 and 555 at dilutions of 1:100. Cells were examined using the 63x oil immersion objective. FRET was assessed using acceptor photo-bleaching approach according to A. K Kenworthy (Kenworthy, 2001). Briefly, cellular regions were selected and photo-bleached using a pre-tested protocol saved in the 710 LSM confocal microscope to achieve a ~60% reduction in fluorescence in the acceptor channel (Ezrin-A555). As a negative control, an intracellular region was collected and photo-bleached in each field. In addition, a peripheral region without photo-bleaching was collected for the calculation of photo-damage in each field. The fluorescence intensities for both channels from 89 selected regions were recorded and saved for later analysis. The FRET efficiency for Dsg3-A488 channel was calculated in an Excel spreadsheet as the percentage increase of donor fluorescence, with photo-damage to be taken into account, after photo-bleaching of the acceptor (Ezrin-A555) by the following formula (Tsang, et al., 2010):

$$FRET = \left(\frac{(fluorescence_{POST} + photo\ damage)}{(fluorescence_{PRE})} \right) - 1 \times 100$$

Regions with FRET efficiency greater than 5% (threshold) were considered to be positive and are presented as mean±SD in Chapter 5.

2.4.6 Proximity ligation Assay (PLA)

The proximity ligation assay allows the detection of molecular interaction within 10 nm. As illustrated in Figure 2.3A, two target molecules are identified with primary antibodies raised in different species, for example, rabbit and mouse. Proximity ligation probes, anti-mouse and anti-rabbit are then added, which have conjugated oligonucleotides (Figure 2.3B). Upon addition of ligase, ligation of the oligonucleotides conjugates will only occur when the complementary proximity probes are within 10-40 nm distance (Figure 2.3C). Successful ligation provides a circular template. Upon the addition of polymerase and nucleotides rolling-circle amplification (RSA) occurs to generate a concatemeric product, which is detected by the addition of labelled oligonucleotides which hybridise to the RSA product (Figure 2.3D). The subsequent interactions can be identified, by fluorescent microscopy, as a single signal or 'blob'. In preparation, cells were seeded onto glass coverslips to achieve sub-confluence following by overnight incubation. Cells were then fixed with ice cold Methanol:Acetone as described previously. The PLA assay was carried out in

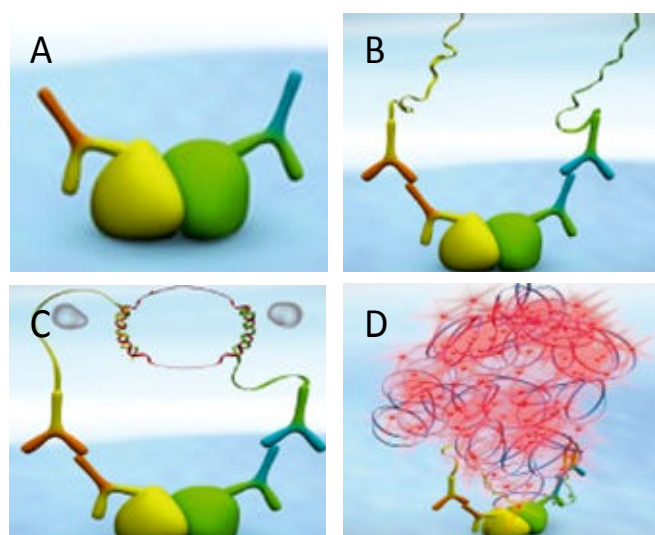


Figure 2.3. Proximity Ligation Assay. (A) Target proteins identified by primary antibodies. (B) Proximity Ligation probes conjugated with complementary oligonucleotides detect the primary antibodies. (C) Only when the probes are within 10-40nm will the ligation of oligo conjugates take place to form a closed circle template. (D) The addition of polymerase and nucleotides allows rolling circle amplification to occur, the product of which is detected as the fluorescence labelled oligonucleotides.

accordance to the guidelines provided by Olink (Cambridge Bioscience) using blocking buffers, antibody diluents, antibody concentrations and incubation periods optimal for the primary antibodies used in fluorescence microscopy. Images were obtained using Zeiss 710 LSM confocal microscope. Image analysis was carried out using ImageJ to determine average dots per cell (Analyse>Analyse Particles) and presented as mean dots/cell \pm SD.

2.4.7 Human Phospho-Kinase Array

A431-Vect Ct and -D3 cells were seeded to achieve sub-confluence in 10cm tissue culture plates and allowed to propagate for 5 hours. Total cell lysates were then harvested and 500 μ g protein was analysed immediately with R&D Proteome Profiler™ Array according to the manufacturer's specifications. In parallel, A431-D3 cells with Dsg3 knockdown were also examined. The dot blots generated from the array displayed 46 kinases, each in duplicate. The density of each spot was determined using ImageJ. Values for each kinase were averaged and then background subtracted. Finally, the Log2 ratio of Vect Ct:D3 was calculated (see below) which allows for the identification of 2 fold variations in kinase phosphorylation in the Dsg3 overexpressing and knockdown cells with respect to their matched control.

$$Log^2ratio = Log^2 \left(\frac{Test}{Control} \right)$$

2.5 Functional assays

2.5.1 Scratch assay

Scratch assay: manual

Cells were seeded in a 6-well tissue culture plate to achieve a confluent monolayer following incubation overnight. Normal growth media was then replaced with growth media supplemented with Mitomycin C (4ug/ml) to inhibit cell proliferation and incubated for a further 4 h. The confluent monolayer was washed with PBS and scratched in a standardised manor using a P200 pipette tip to create a cell-free zone (wound) approximately 3 mm in width. Cells were washed further with PBS to remove any floating cells and cellular debris before addition of fresh growth media supplemented with Mitomycin C for the duration of the experiment. Images were acquired at various time points using the Nikon Eclipse TE2000-S inverted microscope. Cell migration was assessed by determining percentage wound closure of the cell-free zone using ImageJ software.

Scratch assay: IncuCyte™

Cell migration was further assessed by scratch-wound assay using IncuCyte™, a Kinetic Imaging System (Essen BioScience Inc., MI, USA). Here cells were seeded into 96 well image-lock plates in duplicate (Essen BioScience Inc., MI, USA) at 80% confluence to achieve a confluent cell monolayer after 24 hours. The cell-free zone (wound) was then created using the 96-well Woundmaker™ (Essen BioScience Inc., MI, USA). Cells were washed 3 times with PBS to remove any cellular debris followed by the addition of 100 µl growth media. Finally, the image lock plates were loaded into the IncuCyte™ which was programmed to acquire an image from each well every 15 minutes for a time period of 28 hours. The data was analysed using the Relative Wound Density (RWD) metrics which is part of the IncuCyte software package, requiring the area of the wound and the cellular monolayer at either side of the wound at time zero to be defined. Based on these parameters the software determined RWD by measuring the

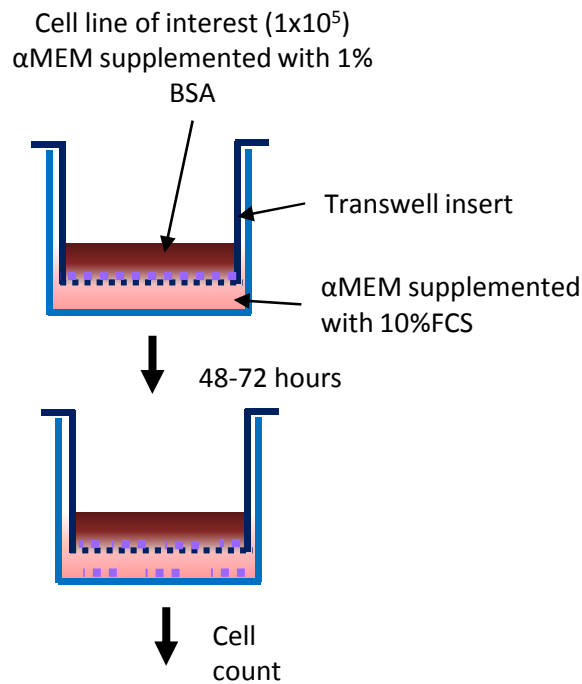


Figure 2.4. Transwell migration assay. A simplified schematic of the transwell migration assay. As depicted, cell line of interest, suspended in α MEM supplemented with 1% BSA, is added into the transwell insert. The medium containing 10% FCS in the bottom chamber acts as a chemottractant. Cells are migrating through the porous membrane of the insert into the bottom chamber. After 24-48 hours, the number of cells that have migrated into the bottom chamber are counted

spatial cell density in the wound area relative to the spatial cell density outside of the wound at every time point. Finally, the RWD of each duplicate was averaged and presented as Mean RWD.

2.5.2 Transwell migration and invasion assay

Transwell assays were conducted using 24-well transwell inserts with an 8 μ m pore size permeable membrane (VWR international Ltd). As illustrated in Figure 2.4, 1×10^5 cells suspended in alphaMEM supplemented with 1% bovine serum albumin (BSA) were aliquoted into the insert and allowed to settle for 1 hour before the addition of 500 μ l of alphaMEM supplemented with 10% FCS for A431 cells or KGM supplemented with 10% FCS for SqCC/Y1 cells into the bottom chamber of each well. Plates were incubated for 24-48 hours to allow cells to migrate through the membrane of the insert. The number of migrated cells was determined using the CASY cell counter (ROCHE Innovastis). For transwell invasion assay, transwell inserts were coated with a thin layer (75 μ l) of Matigel diluted in DMEM (1:3) which was allowed to gel for 2 h before the addition of cell suspensions as described above. For A431 cells, Matrigel was diluted 1:3 in DMEM supplemented with fibronectin (10 μ g/ml).

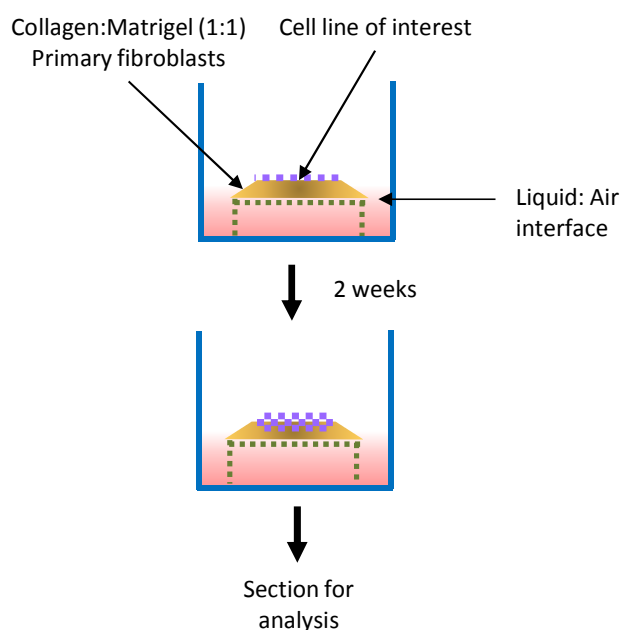


Figure 2.5. Organotypic raft culture. Collagen:Matrigel gels with the embedded primary fibroblasts and cell line of interest plated on top were prepared 24 hours before being lifted on a raft and grown at the air:liquid interface for 14 days before fixation for histological analysis.

2.5.3 Organotypic raft culture

Culture preparation

Matrigel: Collagen gels for organotypic cultures were prepared as described by Thomas, *et al.*, (Thomas, et al., 2001). Briefly, Collagen type 1 (4 mg/ml, Upstate), Matrigel (BD), 10x DMEM (Gibco), FCS and primary fibroblast (Isolated from human forskin) suspension in DMEM+10% FCS at 5×10^5 /ml were mixed at a ratio of 3.5:3.5:1:1:1 on ice. One ml aliquots of this solution were added into wells of a 24-well plate and allowed to gel for 30min at 37°C. Once polymerised 1 ml of DMEM plus 10% FCS was added to each well and gels were allowed to equilibrate overnight at 37°C. For the preparation of nylon discs, sterile nylon discs (100 μ M pore size, Tetko Inc) were coated with collagen gel mixed with the above solution without Matrigel and fibroblasts and incubated for 15 min at 37°C followed by fixation in 1% Glutaraldehyde for 1 h at 4°C. The fixed discs were then washed 3x in PBS and 2x in KGM and stored in DMEM+10% FCS at 4°C until needed. The following day media was aspirated in wells containing the Matrigel: collagen gels and replaced with 5×10^5 SqCC/Y1-Vect Ct or SqCC/Y1-D3 cells suspended in KGM plus 10% FCS and allowed to propagate for 24 h. The next day each gel with SqCC/Y1 cells was removed from the 24-well plate and placed on an individual collagen-coated nylon disc. The gel:disc composite was then placed on a steel grid housed in the well of a 6-well plate. KGM was then added in the

well to reach the under-surface of the grid and epithelial monolayers were allowed to grow at the air:liquid interface (Figure 2.5). This point was defined as day 1. The cultures were incubated at 37°C for 14 days with media changed every 2 days. Steel grids were formed from 2.5cm² stainless steel mesh with its edges bent down to form 4-5 mm legs. Gels were harvested and fixed at day 14 by submerging in Trumps fixative (4% formaldehyde, 1% glutaraldehyde in phosphate buffer, pH adjusted to 7.2) overnight. Specimens were then bisected and embedded in paraffin wax for sectioning (5 µM). Sections were then stained with Haematoxylin and Eosin for the determination of the Invasion index or immunochemically stained with the *mycTag* specific antibody as described below.

Staining of organotypic specimens.

To confirm the overexpression of exogenous hDsg3.*myc*, sections were subjected to routine procedures for histology. Briefly; sections were de-waxed in xylene and brought to absolute alcohol. Antigen retrieval was achieved using ARS for 20 min at 37°C. The detection of exogenous Dsg3 was achieved using rabbit *mycTag* antibody (Cell signalling) in conjunction with Dako cytomation EnVision+Dual Link System-HRP. Sections were counter stained in Mayer's Haematoxylin. Images were acquired in a MBF stereology system (MBF Bioscience, USA).

For Haematoxylin and Eosin staining, sections were de-waxed, rehydrated and placed in Ehrlich's Haematoxylin for 45-60 min. Sections were then rinsed in tap water for 30 s. Sections were examined at this stage and de-stained in 1% HCl in 70% alcohol as necessary to achieve good contrast. Once achieved, they were washed in tap water. Sections were then dehydrated and counter stained with Eosin, dehydrated again before treatment with histoclear for 2 min before mounting. Images were acquired in a MBF stereology system (MBF Bioscience, USA).

Analysis of Organotypic cultures: Invasion index

Images were analysed using ImageJ as described previously (Thomas, et al., 2001). Briefly, images were converted to grey scale (8-bit). Using the threshold the positive stained areas were defined and converted into a binary image which rendered the stained area as saturated red pixels and the unstained, back pixels. This binary image was then subjected to two 'Despeckle' procedures to remove small artefacts. The

invasion distance, number of particles (invaded clusters), average size of particles and sum of the area of particles were then calculated using the 'analyse particles' tool. The invasion index was then calculated by the formula shown below:

$$\text{Invasion index} = \text{Invasion depth} \times \text{number of particles} \times \text{area of particles}$$

ARS (Antigen Retrieval solution)

- 0.1% α -chmotrypsin
- 0.1% calcium chloride

In water, pH adjusted to 7.8.

FGM (Fibroblast Growth media)

DEMEm supplemented with 10% FCS and Glutamine.

KGM (Keratinocyte Growth Media)

- DMEM:F12: 3:1
- 0.4% $\mu\text{g/ml}$ hydrocortisone
- 10 ng/ml EGF
- 10% FCS
- 5 $\mu\text{g/ml}$ insulin

TBS

- 50 mM Tris-Cl buffer, pH 7.5
- 15 mM Sodium Chloride

Trumps fixative

- 100 nM NaH_2PO_4
- 67.5 mM NaOH
- 4% formaldehyde
- 1% Gluteraldehyde

2.7 Statistical analysis

2.7.1 Descriptive statistics

The mean of each test group *i.e.* A431-Vect Ct, -D3, -C7 and -C11 was determined for each repetition of an experiment and presented as the Mean \pm Standard deviation (SD), to give an indication of the variation of the data. Where possible, comparison between control and test groups was normalised against the control and expressed as a fold change \pm SD relative to the control.

$$\text{Mean } (\bar{X}) = \frac{(X_1 + X_2 + \dots + X_n)}{n}$$

$$\text{Standard deviation (SD)} = \sqrt{\frac{\sum (X - \bar{X})^2}{n}}$$

2.7.2 Inferential statistics

The statistical significance of differences between test samples and controls was achieved using the unpaired, 2 tail student's *t*-Test, as data was assumed to be normally distributed and that the overexpression of Dsg3 had neither a positive or

negative effect on the parameters being tested. The student's *t*-Test was calculated using the formula in Excel for a 2 tailed test on 2 samples of equal variance. P values less than 0.05 were considered statistically significant, *i.e.* * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2.6 Antibodies

Table 2.3 Antibody used for Western blot analysis and immunofluorescence microscopy.

Target	clone		Host species	Manufacturer	W/B [working]	IMF [working]
Dsg3	5H10 (Sc-23912)		mouse	Santa Cruz	1:1000	1:100
Dsg3	H145		rabbit	Santa Cruz	1:1000	1:100
Ezrin			mouse	BD	1:1000	1:100
Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558)	pERM		rabbit	Cell signalling	1:1000	1:500
Ezrin	3145	pAb	rabbit	Cell signalling	1:1000	1:100
Radixin			mouse		1:1000	1:100
Moesin (aa554-564)					1:1000	1:100
CD44			mouse		1:1000	1:100
beta Actin-loading control	ab4074		rabbit	Abcam	1:1000	N/A
PKC (aa373-672) of PKC α	A3 Sc-17769	mAb -	mouse	Santa Cruz	1:1000	1:100
Myc-Tag	71D10	mAb	rabbit	Cell signalling	1:1000	1:50
Dsg2	G129	mAB	mouse	Progen	1:50	N/A
Dsg1	P-124	mAB	mouse	Progen		
GAPDH	14c10	mAb	rabbit	Cell signalling	1:1000	N/A
Tubulin	Ab7291		mouse	Abcam	1:1000	N/A
cJun		mAb	rabbit	Cell signalling	1:1000	1:100
Phosph-c-Jun (S73)		mAb	rabbit	Cell signalling	1:1000	1:100
Phosph-c-Jun (Ser63)	54B3	mAb	rabbit	Cell signalling	1:1000	1:100
Phospho-p38 MAPK	28B10	mAb	mouse	Cell signalling	1:1000	1:100
Phospho-PKC (pan) (β II Ser660)	9317	pAb	rabbit	Cell signalling	1:1000	1:100

Table 2.3 continued...

Table	clone		Host species	Manufacturer	W/B [working]	IMF [working]
Anti-rabbit Alexa Fluor®488			goat	Invitrogen	N/A	1:100
Anti-mouse Alexa Fluor®488			goat	Invitrogen	N/A	1:100
Anti-rabbit Alexa Fluor®568			goat	Invitrogen	N/A	1:100
Anti-mouse Alexa Fluor®568			goat	Invitrogen	N/A	1:100
Phospho-STAT Sampler kit 9914.			All rabbit	Cell signalling	All 1:1000	N/A
pSTAT1 (Tyr701)	7649	mAb				
pSTAT2 (Tyr690)	4441	pAb				
pSTAT3 (Tyr705)	9145	mAb				
pSTAT3 (Ser727)	9134	pAb				
pSTAT5 (Try694)	9359	pAb				
pSTAT6 (Try641)	9359	pAb				
	9361	pAb				
Integrin Sampling Kit (611435)			All mouse	BD		
Fibronectin	610077				1:5000	
Integrin α2	611016				1:250	
Integrin α3	611044				1:250	
Integrin α5	610633				1:5000	
Integrin αL	610826				1:500	
Integrin αV	611012				1:250	
Integrin β1	610467				1:2500	
Integrin β3	611140				1:2500	
Integrin β4	611232				1:250	

Chapter 3: **Overexpression of full length human Desmoglein 3 in the A431 epidermoid cancer cell line.**

3.1 Introduction

Previous studies have used one or a combination of models, including primary tumour cells, cancer cell lines, stable knockdown cells, xenograft and allograft models and transgenic mice (Baron, et al., 2012, Chen, et al., 2013, Merritt, et al., 2002) to examine the functions of Dsg3 in the epidermis, the autoimmune disease PV and cancer. As animal work was not possible for this project, an *in vitro* cancer cell model was employed, using the epidermoid A431 cell line overexpressing full length human Dsg3. Using this approach, this study is the first of its kind to adopt a gain-of-function approach to examine the role of Dsg3 in cancer cell biology.

There are a multitude of *in vitro* methods that can be adopted to examine protein function. In principle, they fall into two categories: 1) a gain-of-function approach achieved through the overexpression of target gene(s) or through the constitutive activation of the protein of interest through a gain of function mutation; 2) a loss-of-function approach achieved through protein silencing by RNAi or the expression of dominant negative protein to reduce or abolish protein function. All these methods have their advantages and disadvantages as the manipulation of cellular genetic material, protein structure and expression may have biological effects not directly pertaining to the protein of interest resulting in misleading observations. By using a

combination of techniques one can compensate for some of the limitations of a single model for the examination of protein function.

This chapter aims to characterise exogenous protein expression in the A431 epidermoid cancer cell model, with both gain- and loss-of-Dsg3 function. Employing both qualitative and quantitative techniques, verification analyses of this model system was essential for the later functional and molecular study of Dsg3 in tumour cell biology, including migration and invasion, morphological changes and the mechanistic investigations.

3.2 Results

3.2.1 Stable overexpression of full length human Dsg3 in A431 cancer cell line

In order to examine protein function, stable overexpression is a common practice. In this study, full length human Dsg3 with *myc*Tag (hDsg3.*myc*) was transduced into the A431 cancer cell line. The hDsg3.*myc* construct was generated, for the first time, by Dr Hong Wan at the Institute of Dentistry, QMUL in 2007, providing a unique tool to study the function of Dsg3 (Tsang, et al., 2010).

As illustrated in Figure 3.1, the hDsg3.*myc* construct was cloned into the pBABE retroviral vector containing a puromycin resistant gene to facilitate selection of positively transduced cells. The vulva carcinoma cell line A431 and oral carcinoma cell line SqCC/Y1 were transduced with pBABE-hDsg3.*myc* alongside the empty vector which served as a control (pBABE-Vect Ct). Cells were infected with retroviral conditioned media for 48 h followed by selection for 2 weeks in growth media containing puromycin. From this point onwards, transduced A431 and SqCC/Y1 cell lines expressing hDsg3.*myc* or empty Vector will be followed by the suffix -D3 or -Vect Ct, respectively.

To characterise the expression and localisation of exogenous hDsg3.*myc*, several methods were employed. Firstly, Western blot of total cell lysates from A431-D3 and -Vect Ct cells seeded at 50-60% confluence for 24 h were probed with Dsg3 specific antibody, 5H10, which recognises an extracellular epitope located within the EC1-EC2 domains of Dsg3 (Proby, et al., 2000). As illustrated in Figure 3.2A, the expression of exogenous hDsg3.*myc* construct in A431 cells resulted in a significant increase (~4-fold) in Dsg3 expression compared to endogenous Dsg3 in Vect Ct cells. Secondly, to determine the localisation of exogenous Dsg3, fluorescence microscopy of A431 cells was performed. In preparation, cells were cultured on coverslips to achieve 50-60% confluence following overnight culture. The following day cells were fixed and immunostained for Dsg3 using 5H10, H145, which recognises a intracellular C-terminal epitope of Dsg3 (Lee, et al., 2009), and *myc*Tag specific antibodies. As shown in Figure 3.2B, there is a noteworthy increase in Dsg3 and *myc*Tag staining in A431-D3 cells compared to Vect Ct cells. Quantitation of 5H10 staining alone demonstrated a ~15-fold increase in Dsg3 expression compared to the Vect Ct (Figure 3.2C). With regard to protein localisation, Figures 3.2A & B clearly show that the increased Dsg3 staining was

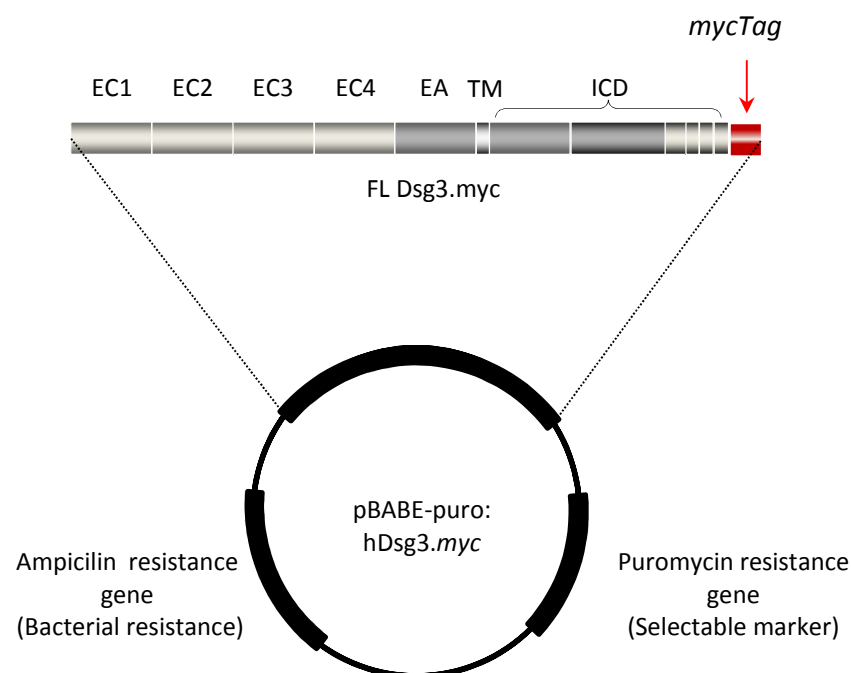


Figure 3.1 Full length human Dsg3 construct in pBABE-puro retroviral vector. Simplified schematic of full length human Dsg3 with *mycTag* (*hDsg3.myc*) construct cloned into the pBABE-puro retroviral vector. pBABE-puro vector contain 2 markers, i.e. the Ampicillin resistance gene that allows the selection of transformed bacteria, and the Puromycin gene for the selection of positively transduced cells.

predominately concentrated at the cell borders and cell-cell contacts as expected. Together, the data confirmed the overexpression of exogenous Dsg3 in A431-D3 cells. Furthermore, these results also demonstrate that the commercially available human Dsg3 antibodies, 5H10 and H145, recognise the *hDsg3.myc* construct. Finally, the exogenous Dsg3 appears to be functioning normally due to its expression and subsequent localisation at the plasma membrane alongside endogenous Dsg3.

3.2.2 Sub-cloning of A431-D3 cells.

The transduction of full length human Dsg3 into A431 cells generated a mixed population of cells (polyclonal) with a wide range of exogenous Dsg3 expression levels. In order to demonstrate a dose-dependent response of phenotypes observed, the variation of Dsg3 expression of polyclonal A431-D3 cells was used to an advantage. Sub-cloning of A431-D3 cells was performed by limited dilution assay (courtesy of Dr Tsang) to yield a panel of individual clones expressing high or low Dsg3 expression. This procedure should theoretically yield clones which display uniform Dsg3 expression compared to A431-D3 cells. Florescence microscopy illustrated in Figure 3.3B (n=5) demonstrates the high Dsg3 expression of A431-C7 clone and low expression of A431-

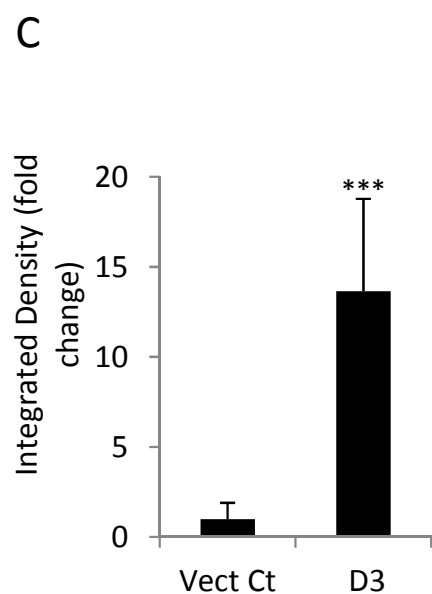
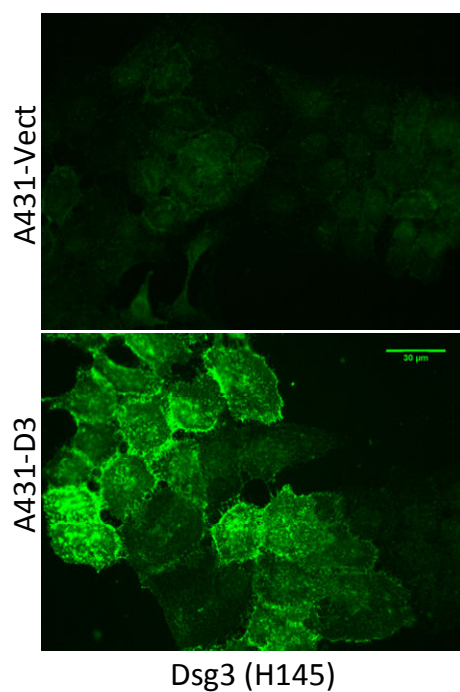
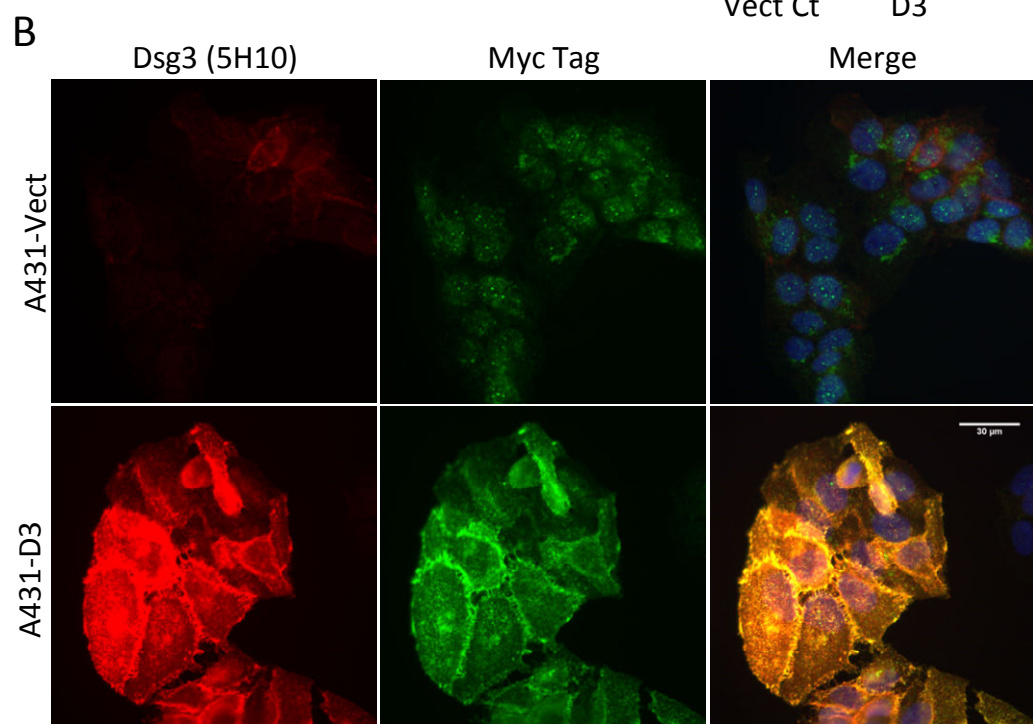
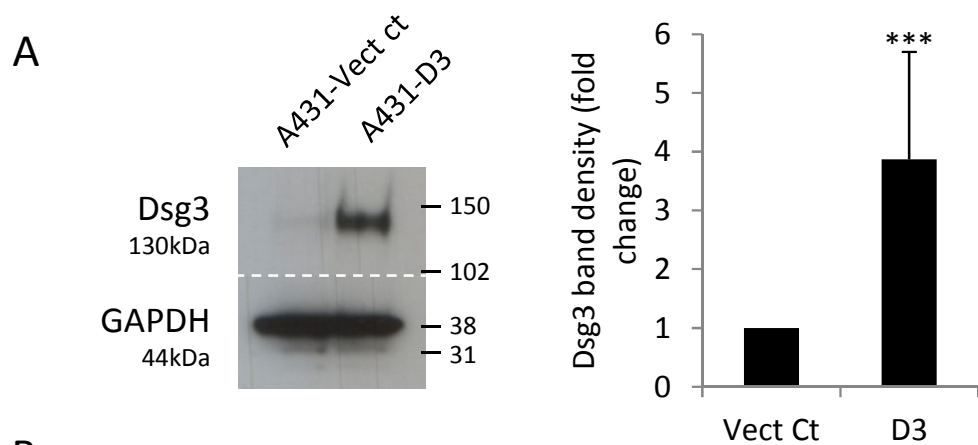


Figure 3.2 Stable overexpression of full length human Dsg3 in A431 cells. (A) To assess the expression of the hDsg3.myc construct in A431 cells, Western blot of total cell lysates of A431-D3 and Vect Ct cells was performed with the mouse monoclonal antibody 5H10 (n=9). Blot densitometry: normalised against GAPDH and relative Vect control level set at 1 arbitrary unit. Dotted line indicates where parts of the same blot, of either the same or different exposure, was joined together. **(B)** Fluorescence micrographs of A431-D3 and Vect Ct cells stained with the Dsg3 antibodies, 5H10 (extracellular epitope) and H145 (intracellular epitope) as well as the mycTag specific antibody. Increased Dsg3 expression was readily observed in D3 cells compared to Vect Ct. **(C)** Quantitation of 5H10 staining in A431-D3 and Vect Ct cells (n=10, ***P<0.001). Scale bar 30 µm.

C11 clone. Western blot analysis (n=7) demonstrated a ~4-fold increase in A431-D3 and C7 band density compared to that of Vect Ct and C11 cells (Figure 3.3A). In support, analysis using absolute quantitative Reverse Transcription PCR (n=2) demonstrates a ~20- and ~30-fold increase in Dsg3 mRNA transcription in A431-D3 and C7, respectively, compared to Vect Ct and C11 cells (Figure 3.3C). For the absolute quantification of Dsg3 mRNA using real-time RT-qPCR as a means of assessing Dsg3 transcription, mRNA was extracted from A431 -Vect Ct, -D3, -C7 and -C11 cells seeded at ~50-60% confluence 24 h prior to extraction. cDNA was generated by reverse transcription reactions from the mRNA transcripts recovered. The cDNA samples provided the template for real time qPCR reactions for target genes alongside the reference genes POLR1 and YAP1. The absolute Dsg3 mRNA copy number was determined by normalising against the reference genes followed by comparison to a Dsg3 standard curve. Both Western blot and fluorescence microscopy was performed and analysed as described above.

In summary, the data shows the significantly high expression of exogenous Dsg3 in A431-D3 and -C7 cells compared to Vector Ct and C11 cells, of both transcriptional and translational levels.

3.2.3 Overexpression of Dsg3 does not appear to affect Dsg1 or Dsg2 expression.

There are seven desmosomal cadherins identified in man, Dsg 1-4 and Dsc 1-3, which share a common function in the mediation of cell-cell adhesion within desmosomes. As a result, a degree of functional redundancy amongst this group of cadherins has been proposed (Brennan, et al., 2010). However studies have highlighted some specific characteristics and functions among desmosomal cadherin isoforms (Garrod, et al., 2002, Ishii, 2003). Desmosomal cadherin isoforms have been shown to adopt cell type and differentiation-specific expression patterns within stratified epithelia with Dsg3

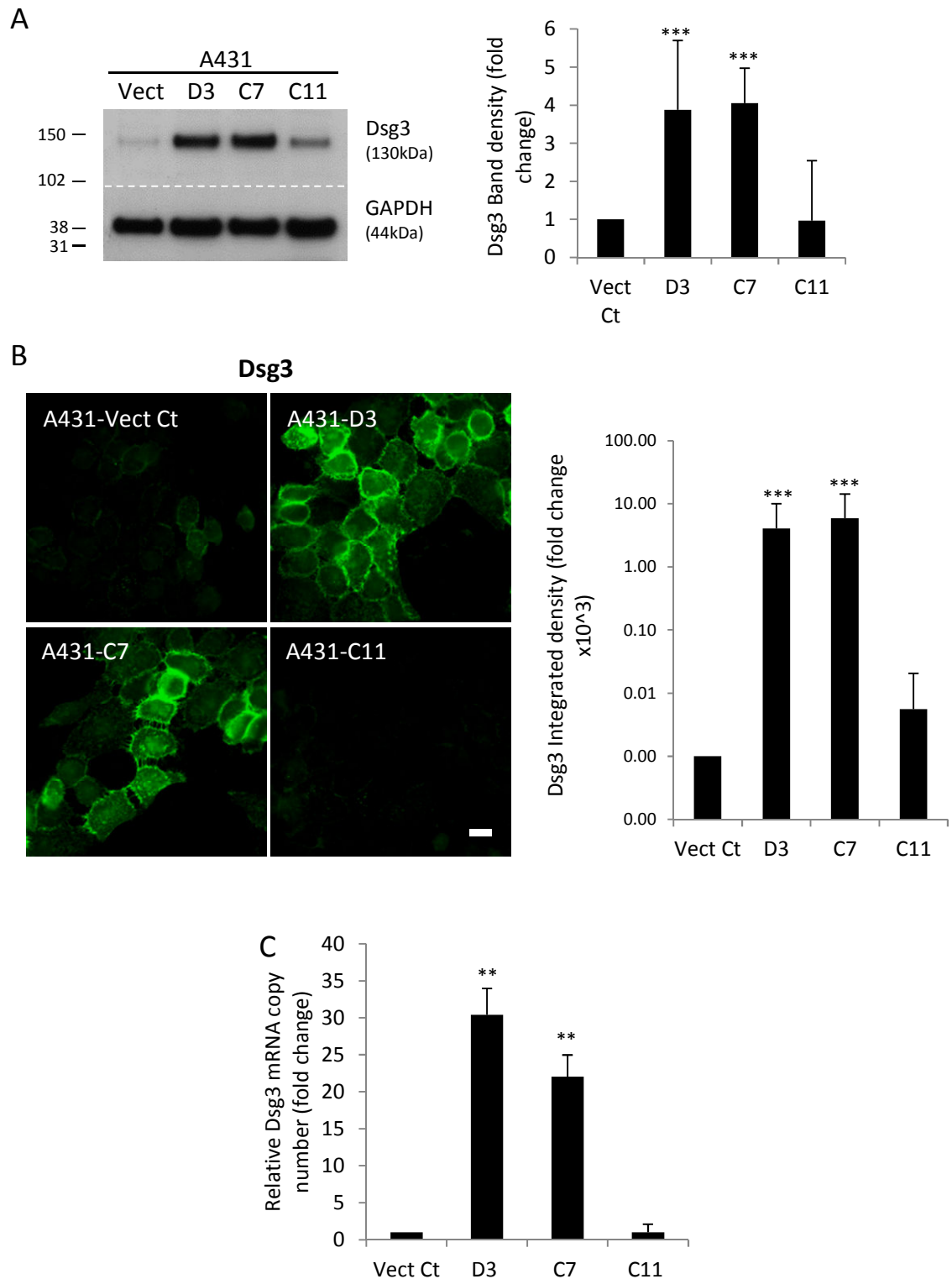


Figure 3.3 Desmoglein 3 expression in A431-D3 clones. (A) Western blot of A431-Vect Ct, D3, C7 and C11 cells with high or low Dsg3 levels, probed with Dsg3 specific antibody 5H10. Bar chart on the right shows fold change in blot band density ($n=7$, mean \pm SD). Dotted line indicates where parts of the same blot, of either the same or different exposure, was joined together (B) Fluorescence microscopy of four cell lines shown in (A) stained with 5H10. Bar chart shows fold change in immunofluorescent staining ($n=5$, mean \pm SD) Scale bar 20 μ m. (C) Relative Dsg3 mRNA copy number in A431-Vect Ct, D3, C7 and C11 lines as determined by qPCR ($n=2$, *** $p<0.001$, ** $p<0.01$). Blot densitometry: normalised against GAPDH and relative Vect control level set at 1 arbitrary unit.

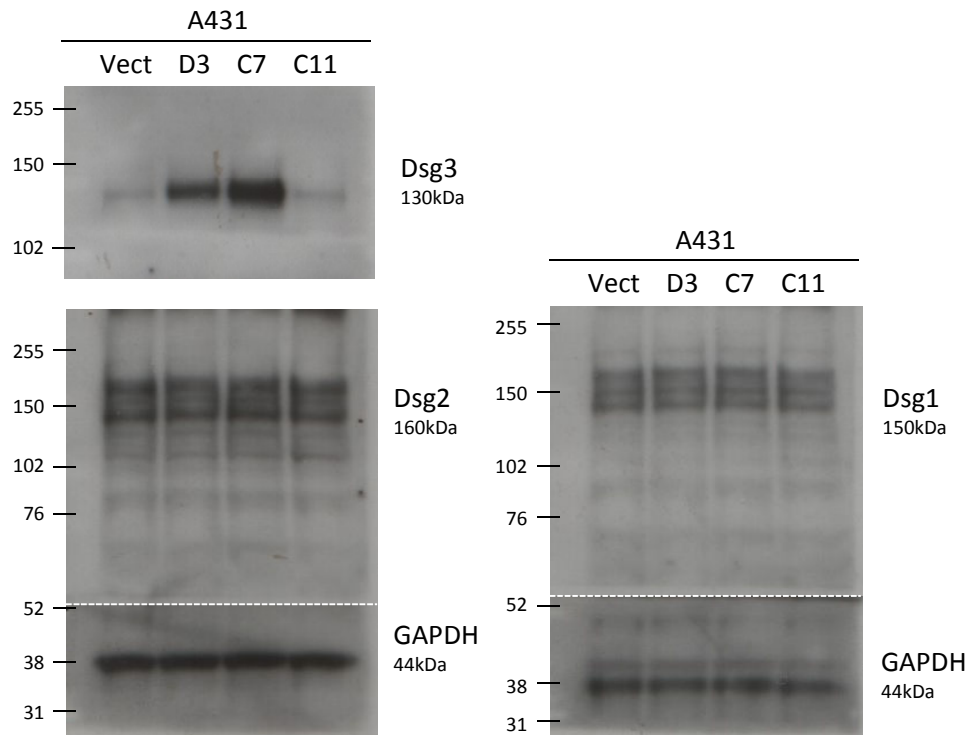


Figure 3.4 Overexpression of Dsg3 does not appear to affect Dsg1 or Dsg2 expression. Western blotting analysis of total cell lysates of A431-Vect Ct, D3, C7 and C11 lines for Dsg1, Dsg2 and Dsg3 (n=2). There appears to be no significant affect on Dsg1 and Dsg2 expression in response to Dsg3 overexpression. Dotted line indicates where parts of the same blot, of either the same or different exposure, was joined together.

being highly expressed in proliferating epidermal layers of the basal and immediate supra-basal layers of the epidermis while Dsg1 is expressed in the upper layers of the epidermis and considered an effective marker for keratinocyte differentiation (Amagai, et al., 1996, Arnemann, et al., 1993, Szegedi, et al., 2009). It has been suggested that these expression patterns are the consequence of PKC induced terminal differentiation of keratinocytes (Szegedi, et al., 2009). One such study carried out by Elias *et al* demonstrated that by altering the Dsg3 expression profile in the epidermis to resembling that of the oral mucosa, *i.e.* uniform expression of Dsg3 across the epithelium, resulted in histological and phenotypic characteristics consistent with the oral mucosa (Elias, et al., 2001). Similarly, overexpression of Dsg3 in the suprabasal layers of the epidermis in transgenic mice elicited hyper-proliferation and abnormal differentiation (Merritt, et al., 2002). Studies examining PV pathogenesis and the compensation hypothesis propose that Dsg1 is able to compensate for the loss of Dsg3 adhesion and visa-versa (Brennan, et al., 2010, Mahoney, et al., 1999). In summary, these reports suggest that desmosomal cadherins confer cooperative and

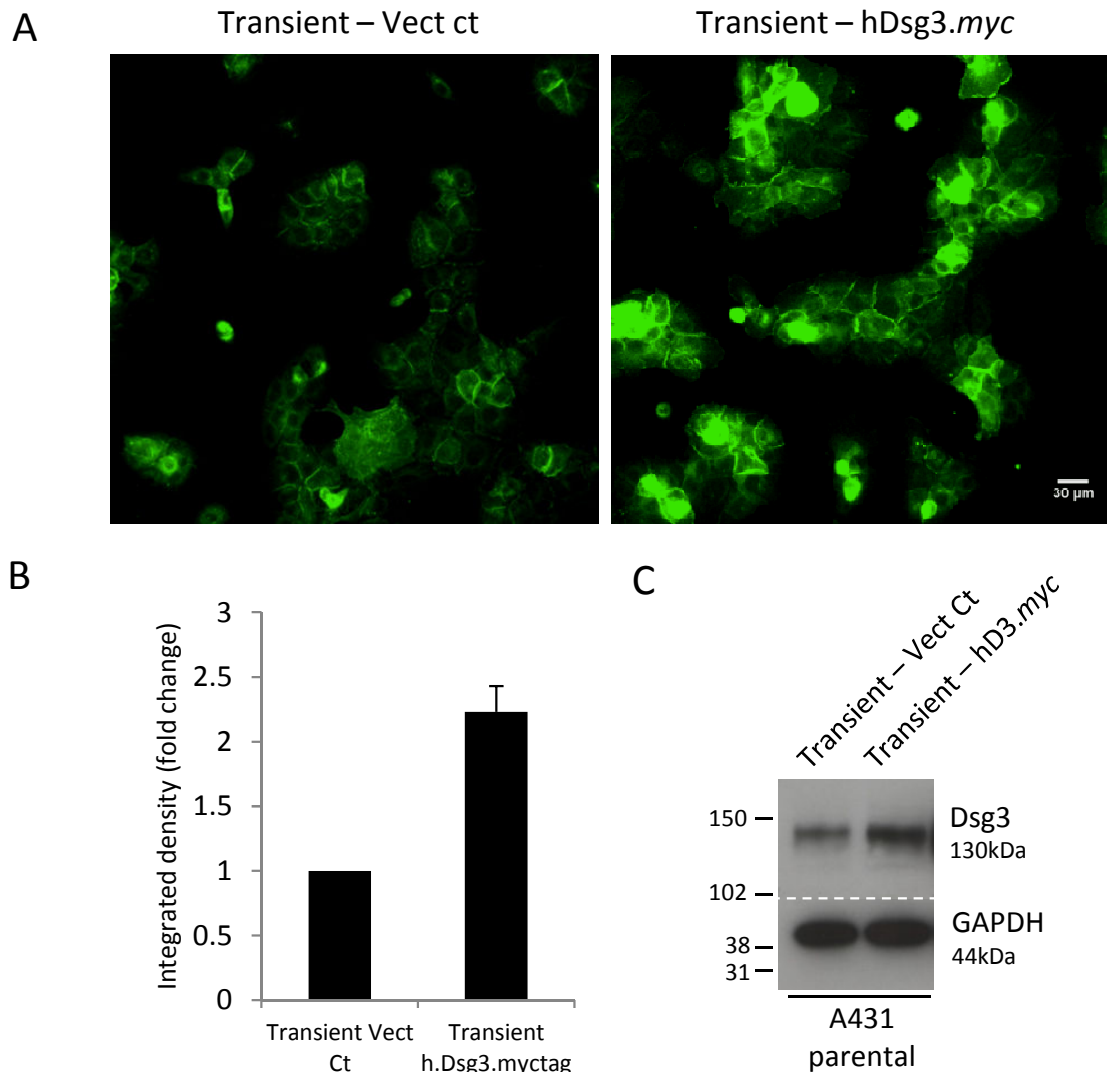


Figure 3.5 Transient overexpression of Dsg3 in A431 parental cells. (A) Fluorescence microscopy of A431 parental cells transiently transfected with empty vector (pBABE-puro) or hDsg3.myc construct and immunostained with the Dsg3 antibody, 5H10. (B) Quantitation of Dsg3 staining in (A) (n=2), Scale bar, 30 μ m. Blot densitometry: normalised against GAPDH and relative Vect control level set at 1 arbitrary unit (C) Western blot of A431 parental cells transiently transfected with hDsg3.myc or empty vector (pBABE-puro) construct. Blots were probed with the Dsg3 antibody, 5H10, and GAPDH as a loading control. Dotted line indicates where parts of the same blot, of either the same or different exposure, was joined together.

compensatory functions in epithelial cells as well as isoforms specific functions. As a result an important question was to determine whether the overexpression of exogenous Dsg3 affects the expression of other desmosomal cadherins, especially Dsg1 and Dsg2. To address this question, Western blot of total cell lysates of A431 cell lines was performed as described previously with the anti-Dsg1, Dsg2 and anti-Dsg3 specific antibodies. As illustrated in Figure 3.4, the overexpression of Dsg3 appeared to have no significant effect on the expression of Dsg1 and Dsg2 compared to the vector control.

3.2.4 Transient overexpression of Dsg3 in A431 parental cells

While retroviral transduction of hDsg3.*myc* into A431 cancer cell lines is a cost effective means of studying protein function, the random integration of the construct into the host genome may elicit effects not attributed to Dsg3 overexpression. As a result, transient transfection of hDsg3.*myc* was also carried out to substantiate some observations made using stable overexpression cell lines. To access the transient overexpression of Dsg3 various techniques, as described above, were employed. In preparation, A431 parental cells were seeded at ~30% confluence 24 h prior to transient transfection with hDsg3.*myc* and empty vector cDNA. The following day cells were re-seeded at the desired cell density for assay 48 h after transfection. As illustrated in Figure 3.5, the transient overexpression of full length Dsg3 in parental A431 cells resulted in a ~2-fold increase in Dsg3 expression, assessed by Western blot (Figure 3.5C). This observation was supported by fluorescence microscopy that demonstrated a significant increase (~2 fold) in Dsg3 staining compared to cells transfected with Vect Ct (Figure 3.5A, B).

3.2.5 Transient knockdown of Dsg3 in A431-D3 cells.

In order to verify the observations from stable and/or transient Dsg3 overexpression, loss-of-function experiments were also performed by transient siRNA transfection. It was expected that Dsg3 knockdown in A431-D3 cells would restore the phenotypes similar to that of Vect Ct cells. Knockdown studies were carried out on A431-D3 or -C7 cells and compared to cells transiently transfected with scrambled Ct siRNA. In preparation, cells were seeded at 30% confluence the day before transfection with Dsg3 specific and scrambled siRNA. The following day cells were harvested and re-seeded at the desired cell density for assay to be carried out 72 h after initial seeding. As illustrated in Figure 3.6, Western blot analysis showed a 5-fold reduction in Dsg3 band density in knockdown cells compared to control cells (Figure 3.6B). This observation was further supported by fluorescence microscopy analysis which showed a significant reduction in Dsg3 staining in A431-D3 cells treated with Dsg3 specific siRNA compared to those treated with scrambled control siRNA (Figure 3.6A).

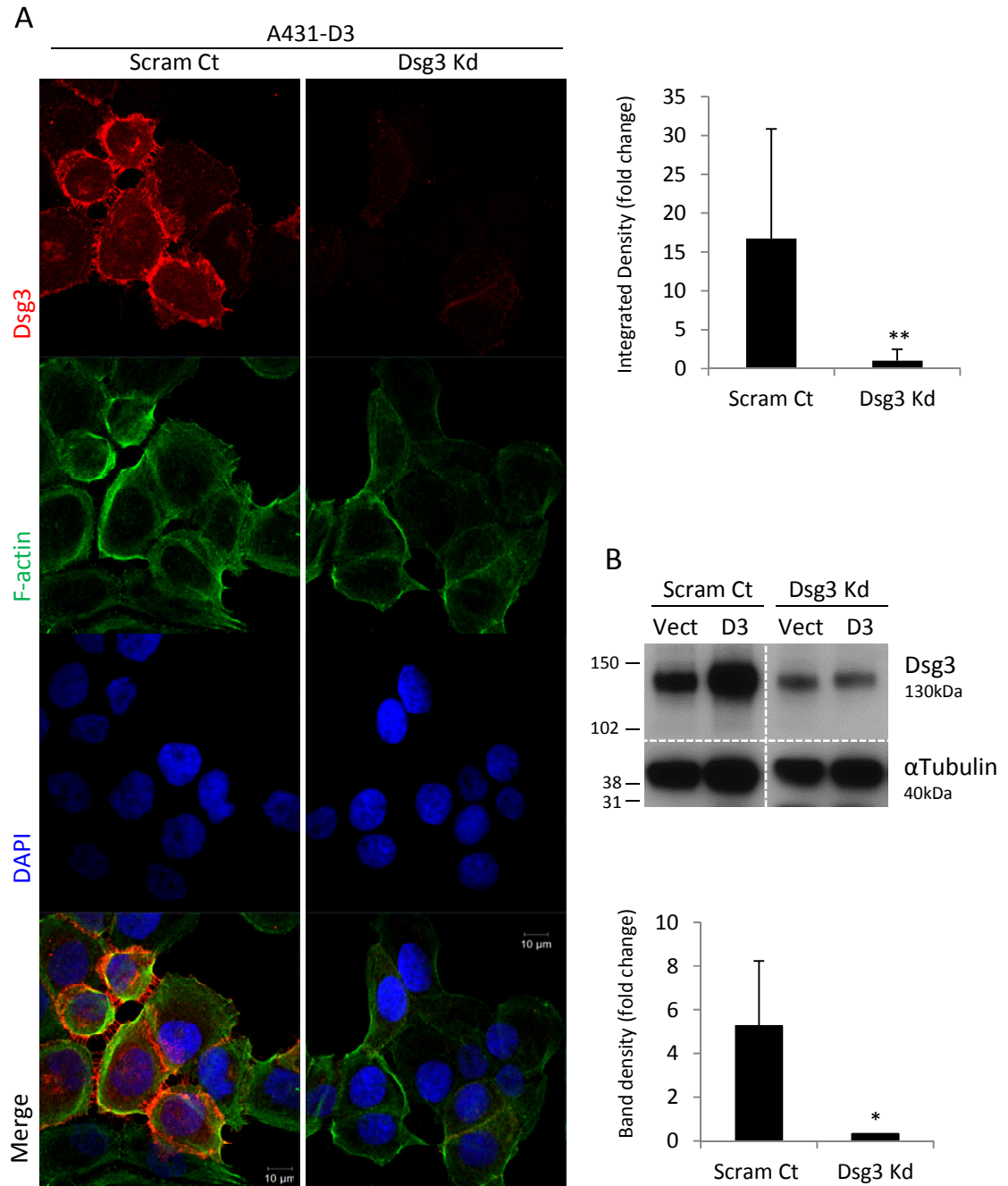


Figure 3.6 siRNA mediated Dsg3 silencing in A431-D3 cells. (A) Fluorescence micrographs of A431-D3 cells transiently transfected with either Dsg3 specific or scrambled control siRNA for 2 days. Quantitation on the right shows mean fold change of the Dsg3 staining ($n=2$, mean \pm SD, $**P<0.01$). Scale bar, 10 μ m. **(B)** Western blot of total lysates of A431-Vect Ct and D3 cells transfected with either Dsg3 specific (Kd) or scrambled control (Scram) siRNA. Right: quantitation of A431-D3 cells shows mean fold change in band density. Blot densitometry: normalised against α Tubulin and relative Vect control level set at 1 arbitrary unit ($n=3$, mean \pm SD, $*P<0.05$). Dotted line indicates where parts of the same blot, of either the same or different exposure, was joined together.

3.3 Discussion

This chapter demonstrates the successful stable and transient overexpression of full length human Dsg3 in A431 epidermoid cancer cell line and efficient siRNA mediated Dsg3 silencing. Both overexpression and silencing elicited a detectable and significant increase and decrease in Dsg3 expression by Western blot and immunofluorescent microscopy using commercially available Dsg3-specific antibodies. The overexpression of Dsg3 does not appear to provoke a response from Dsg1 and Dsg2 compared to the vector control. However known positive controls of Dsg1 and Dsg2 expression such as F9 and A-375 cell lysates were not incorporated into this study. As a result it is not clear from the Western blots which bands are Dsg1 and Dsg2 specific. The inclusion of such positive controls would validate the apparent observation reported here. Finally, exogenous Dsg3 appears to be functioning normally due to its correct localisation at the plasma membrane alongside endogenous Dsg3.

Both the loss- and gain- of-function approaches validated in this chapter provide the basis of the investigations described in subsequent chapters, the aim of which is to characterise the additional functions of Dsg3 and elucidate possible signalling mechanisms employed to achieve these functions. It is worth noting that a majority of this study utilises stable Dsg3 overexpression, with transient overexpression and knockdown models used to complement data where necessary.

Due to the random or quasi-random incorporation of full length human Dsg3 into the host genome by retroviral transduction, it would be naïve to assume that all observations were specific to Dsg3 as some could be due to transgene incorporation affecting normal cell behaviours. To substantiate interesting findings and ensure that observations were specific to Dsg3 overexpression additional steps were taken. Firstly, the study was expanded to include stable monoclonal cell lines with high and low Dsg3 expression, A431-C7 and A431-C11 respectively, alongside the mixed cloned A431-D3 and -Vect Ct cells. Secondly, transient overexpression was used in parental A431 cells and finally, Dsg3 silencing mediated by RNAi. By using a combination of approaches the limitation of any one techniques are superseded by advantages of others allowing, with a high degree of certainty to substantiate findings and form valid conclusions.

The overexpression of Dsg3 using transient transfection was not as efficient as the transduction in A431 cells only achieving a 2-fold increase, compared to the 5-fold increase in stable cell lines (Figure 3.4-5). The reduced overexpression of Dsg3 as a result of transient transfection was most likely attributed to the cell system since greater expression was observed by colleagues in COS-1 cells. Further optimisation or an alternative transfection method could be examined for future work to improve transfection efficiency, such as lipid mediated transfection, magnet mediated transfection and electroporation.

It has been reported that Dsg3 deficiency in Dsg3^{-/-} mice results in compensation by Dsg1 and Dsg2 (Baron, et al., 2012) in the Urea soluble fraction or DSM associated cadherins. Furthermore Dsg2 has been shown to compensate for the loss of Dsg1-mediated cell adhesion in PF (Brennan, et al., 2010) suggesting that Dsg1 and Dsg2 may compensate for the loss of Dsg3 expression and function within desmosomes most likely in an adhesive capacity. My results suggest that the overexpression of Dsg3 has no effect on the expression of Dsg1 and Dsg2. It is possible that the overexpression of Dsg3 does not elicit an expressional response from other desmosomal cadherins as there is not a biological need or deficit that requires compensation. The effect of Dsg3 silencing on Dsg1 and Dsg2 expression was not evaluated in this study however, if I were to speculate, I believe the knockdown of Dsg3 in A431-D3 cells would have no effect on Dsg1 and Dsg2 expression since Dsg3 silencing would bring Dsg3 expression to basal levels of expression that would not require Dsg1 and Dsg2 compensation. It is also possible that Dsg1 and Dsg2 may not be able to compensate for Dsg3-specific functions.

Overall the loss- and gain- of function approaches outlined in the chapter provide a suitable means to investigate the functional and mechanistic roles of Dsg3 in tumour cell biology.

3.4 Future work

While a range of *in vitro* gain- and loss-of-function approaches allows confidence in the evidence provided in this thesis, for future development of this project and possible transferable implications, development of this model and the incorporation of others for the study of Dsg3 function would be advantageous.

Retroviral transduction has many advantages such as simple vector design and high transfection efficiency. However, it has the disadvantage of variable site and frequency of genome integration which have been proven to have potentially mutagenic effect (Hacein-Bey-Abina, et al., 2003). Although often viewed as a disadvantage, the random integration also results in unpredictable stability of the transgene which results in varying levels of transgene expression. In our study this 'limitation' was used to an advantage in assessment of Dsg3-dependency. To address some of the limitations of the *in vitro* model the frequency of Dsg3 integration into the host cell genome of single cell clones could be determined using real time qPCR. By comparing the copy number of Dsg3 to a reference gene an accurate measure of insertion frequency can be determined. Subsequently, A431-D3 clones which exhibit a high insertion frequency could be excluded. Other methods that would also be advantageous in an extension of this study include other cancer cell lines characterised as having high Dsg3 expression, such as those used by Chen *et al* in 2007 which include oral carcinoma cells OECMI and SCC-25 and nasopharyngeal carcinoma cells BIM1 and 076 (Chen, et al., 2007). These cells could also be subjected to Dsg3 silencing or transduction with shRNA to generate a stable knockdown cell line. Finally, the use of primary tumour cells identified as over expressing Dsg3, could also be incorporated in future.

Chapter 4: **Desmoglein 3 regulates cell morphology, migration and invasion.**

4.1 Introduction

Changes in cell morphology and the acquisition of cell motility are known to play important roles in a multitude of cellular processes including tissue remodelling, wound repair, differentiation and embryogenesis. Regulators of these processes are essential to maintaining tissue homeostasis. However, aberrant modulation of these regulators is associated with pathological conditions, such as metastasis, by disrupting tissue organisation, increasing cell motility and promoting degradation of the extracellular matrix. One such group of regulators include Rho GTPases (Sahai and Marshall, 2002).

Since the characterisation of seven desmosomal cadherin isoforms (Dsg1-4 and Dsc1-3) questions have been raised, as to the need for so many? And whether they in fact confer unique functionalities? Studies examining functions of desmosomal cadherins have revealed additional roles beyond mediating cell-cell adhesion. Dsg3's morphoregulatory functions are well documented in *in vitro* and in *in vivo* models with altered expression patterns or truncation of Dsg3 resulting in changes in histological and phenotypic characteristics (Elias, et al., 2001), abnormal hyper-proliferation and

differentiation (Merritt, et al., 2002), and inhibition of morphogenesis and cell positioning (Runswick, et al., 2001). Dsg3 has also been shown to be up regulated in cancer, including pulmonary SqCC (Savci-Heijink, et al., 2009), Head and neck SCC, malignant lymph nodes (Wang, et al., 2007) and breast cancer cell lines (Gordon, et al., 2003). Additionally, the level of Dsg3 expression has been reported to correlate with clinical stage of head and neck malignancy (Chen, et al., 2007). Most recently, Dsg3 has been identified as a biomarker for the ultrasensitive detection of lymph node metastasis in oral cancer (Patel, et al., 2013) that led to a patent for its use as a biomarker for lymph node metastasis published in April 2012 (US 2012/008782 A1). Taken together, these findings strongly indicate a regulatory role of Dsg3 in tissue homeostasis, with aberrant expression associated with cancer progression, however, little is known about its precise function in tumour cell biology.

The aim of this Chapter is to investigate the phenotypic effect of Dsg3 overexpression in the A431 cancer cell line with regard to cell morphology, migratory and invasive behaviour.

4.2 Results

4.2.1 Desmoglein 3 enhances cancer cell spreading and membrane protrusion.

Transformation of cells results in diverse changes in cell morphology, proliferation, and adhesion, many of which are prerequisite events to cell migration and invasion. One such morphological characteristic is cell spreading which is facilitated by the extension of filopodia- and lamellipodia-like membrane projections. Preliminary time-lapse microscopy, carried out by Dr Wan (Figure 1.13), suggests that the overexpression of Dsg3 in A431 cells (A431-D3) promotes cell spreading and membrane protrusions compared to Vect Ct cells. To substantiate this observation, fluorescence microscopy was carried out to examine Dsg3 expression and cell area, comparing A431-D3, -C2 (high Dsg3 expression) with vector control cells (A431-Vect Ct). For this experiment cells were seeded at ~60% confluence on coverslips 24 h prior to fixation and staining using Dsg3 specific antibody, 5H10, and phalloidine conjugated with Alexa Flour 488 for F-actin detection. Figure 4.1A illustrates that the level of Dsg3 expression correlated with increased cell area. To confirm this relationship, cell area and Dsg3 expression (integrated density) from two independent experiments were collated. The mean cell area and Dsg3 integrated density per field were then plotted against each irrespective of the specific cell lines used (A431-Vect Ct, -D3, and -C2). As illustrated in Figure 4.1B a positive correlation between the Dsg3 expression and cell area suggests that Dsg3 regulates cell spreading.

With respect to membrane protrusions consistently an increased frequency and exaggerated filopodia-like protrusion were seen in cells overexpressing Dsg3 compared to the Vector control. Illustrated in figure 4.2 are representative images of high expression clones A431-D3, C2 and C7 and Vector control cells seeded a day prior to fixation and staining using Ezrin. As shown the overexpression Dsg3 increases the frequency of membrane protrusions which also appears to more pronounced .To substantiate that the effect of Dsg3 overexpression on membrane protrusions was Dsg3-dependent, Dsg3 knockdown experiments were performed in A431-D3 cells. In preparation cells were seeded 24h prior to transient transfection with Dsg3 specific and scrambled control siRNA. After 24 h, cells were re-seeded at 50-60% confluence on coverslips and grown for a further 24 h before fixation. Cells were then immunostained for Dsg3 (5H10) and Ezrin.

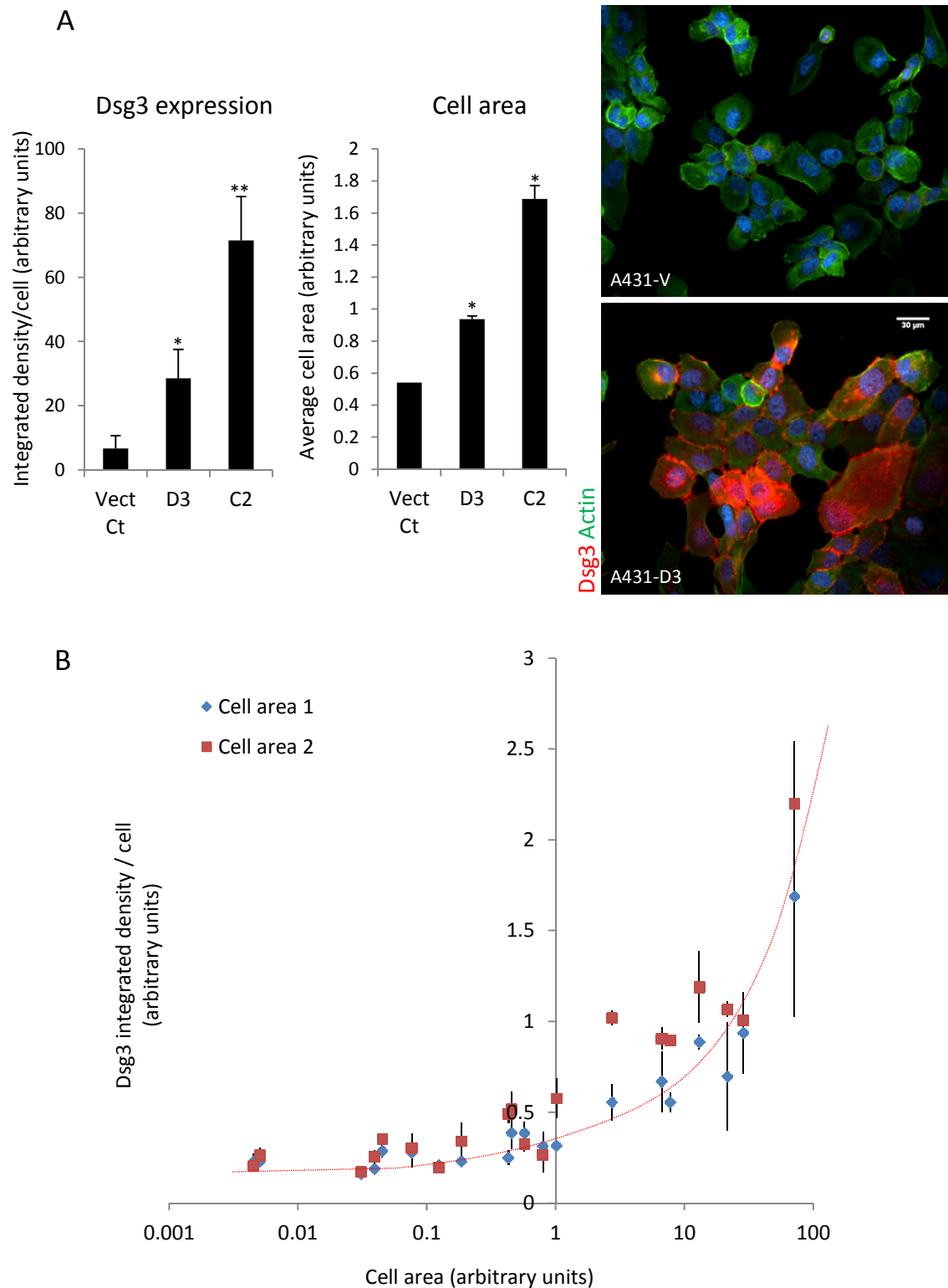


Figure 4.1. Overexpression of Dsg3 increases cell area. (A) A431-V, D3, and C2 cells were seeded at 50-60% density on coverslips and allowed to propagate for 24 h. Cells were fixed and stained for Dsg3 (5H10, red) and F-actin (A488-Phalloidin). Images were acquired using Leica epi-fluorescence microscope. Dsg3 integrated density and cell area in each field were determined using ImageJ and divided by total cell number. At least 3 fields/cell line were analysed in 2 independent experiments. Data presented in bar charts are the average Dsg3 expression and cell area \pm SD (* $P < 0.05$, ** $P < 0.01$). Scale bar 30 μ m **(B)** The correlation between cell area and relative Dsg3 levels per field was plotted from 2 independent experiments (cell area 1, blue diamonds and cell area 2, red squares) (mean \pm SD).

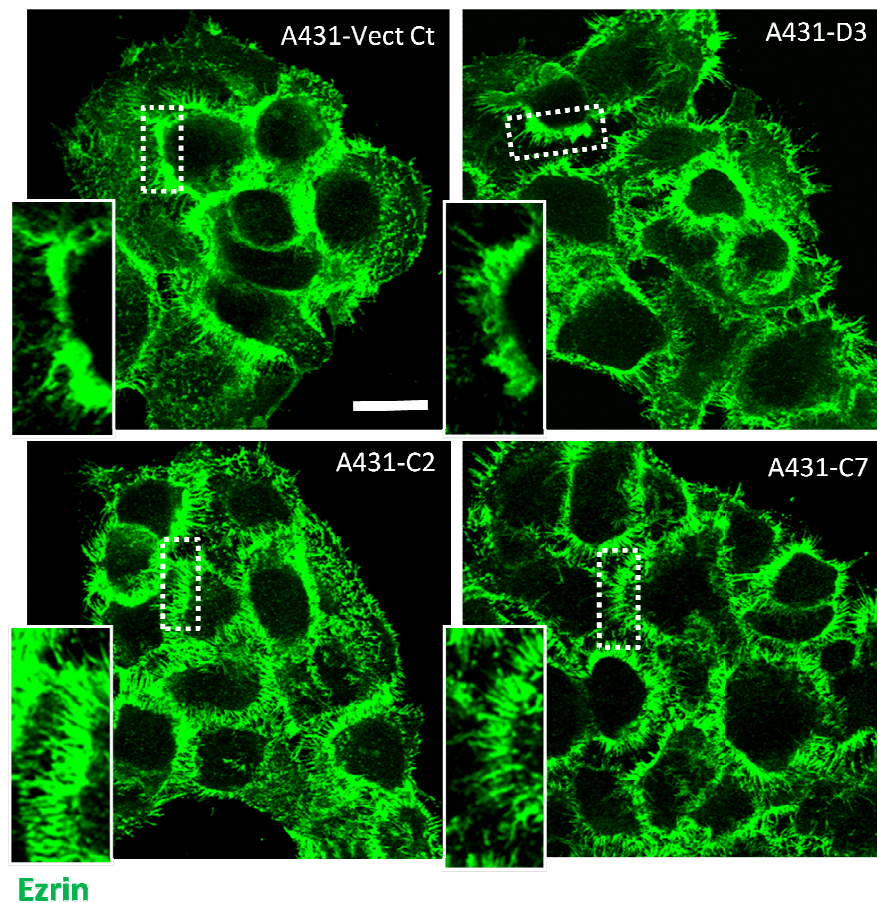


Figure 4.2 Desmoglein 3 overexpression promotes membrane protrusions. (A) Confocal images of A431-V control, D3 polyclonal, C2 and C7 monoclonal cell lines, labelled with the mouse Ezrin antibody. The inserts in each image is the enlarged image of dotted box focusing on membrane protrusions Scale bar, 20 μ m.

As illustrated in Figure 4.3 Dsg3 knockdown in RNAi treated cells resulted in a significant reduction in Dsg3 expression compared to scrambled control. Furthermore, Dsg3 silencing inhibited membrane protrusions (white arrows) compared to control cells, suggesting that the enhanced membrane protrusions in Dsg3 overexpressing cells is Dsg3-specific.

In summary, through both gain- and loss-of-function experimental approaches, it appears that Dsg3 regulates cell morphology by promoting cell spreading and membrane protrusions in A431 cells.

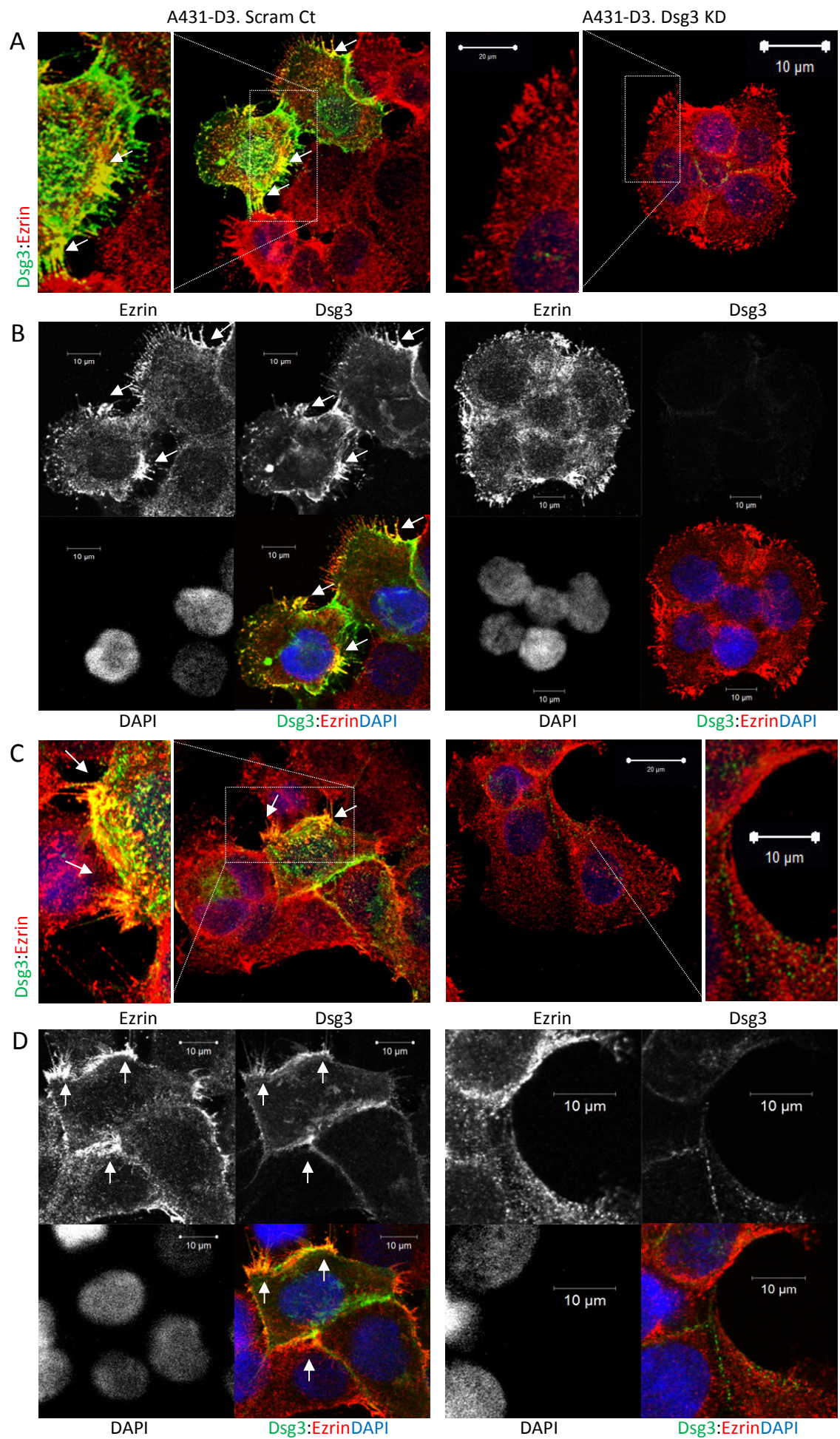


Figure 4.3 Desmoglein 3 silencing inhibits membrane protrusion in A431 cells. (A, B, C & D) A431-D3 cells treated with either scrambled control (Scram Ct: left panels) or Dsg3 specific siRNA (Dsg3 KD: right panels) were fixed and immunostained for Dsg3 (green) and Ezrin (red). **(A)** and **(C)** illustrate merged Dsg3 and Ezrin staining for Scrambled Ct and Dsg3 KD cells. Enlarged images of dotted boxes focus on membrane protrusions, or lack of membrane protrusions (left of square images). **(B)** and **(D)** illustrate the split channels of the images in A & C in black and white. Particular regions of interest (membrane protrusions or lack of highlight with white arrows) The knockdown of Dsg3 resulted in the inhibition of membrane protrusions. Scale bar 20 μ m and 10 μ m, n=2.

4.2.2 Desmoglein 3 promotes cancer cell migration.

A previous study showed that Dsg3 overexpression in A431 cells did not enhance cell-cell adhesion but rather promoted cell migration as assessed by a scratch-wound assay (Tsang, et al., 2010). Furthermore, the data presented thus far suggest that Dsg3 overexpression promotes cell spreading and membrane protrusions as possible prerequisite events of cell migration. To test this hypothesis, various A431 cell lines with different levels of Dsg3 expression were examined by transwell migration and scratch wound assays. The transwell assay of both A431 and SqCC/Y1 were conducted by BSc students, Miss T Mannan and Emina Gunic. In this case, the indicated cell lines were counted and re-suspended in α -DMEM (for A431) or DMEM:Ham's F12 (3:1) (for SqCC/Y1) supplemented with 1% bovine serum albumin. A defined amount of cells were seeded into each 24-well transwell insert. The bottom well was filled with α -DMEM supplemented with 10% FCS for A431 cells or keratinocyte growth medium for SqCC/Y1 Cells. Cells were incubated for 2-3 days to allow migration through the transwell insert into the bottom chamber. The total number of cells that had migrated into the bottom chambers was determined using an automated cell counter. Three independent experiments in triplicate, were performed and demonstrated that the overexpression of Dsg3 in both A431 and SqCC/Y1 cancer cell lines significantly increased cell migration compared to Vect Ct cells and immortalised keratinocyte HaCat cell line which retain normal cell-cell adhesion and served as a negative control (Figure 4.4). Dsg3 silencing in SqCC/Y1 parental cells transduced with Dsg3 specific shRNAs (shRNA-1 and -2), did not show any significant differences compared to Vect Ct cells (V) (Figure 4.4). Cell migration was further assessed by a scratch-wound assay using IncuCyte™, a Kinetic Imaging System which allows for live cell video imaging from within the incubator. In this study, the indicated cell lines were seeded into 96-well image-lock plates at 80% confluence to achieve a confluent monolayer after 24 h. The

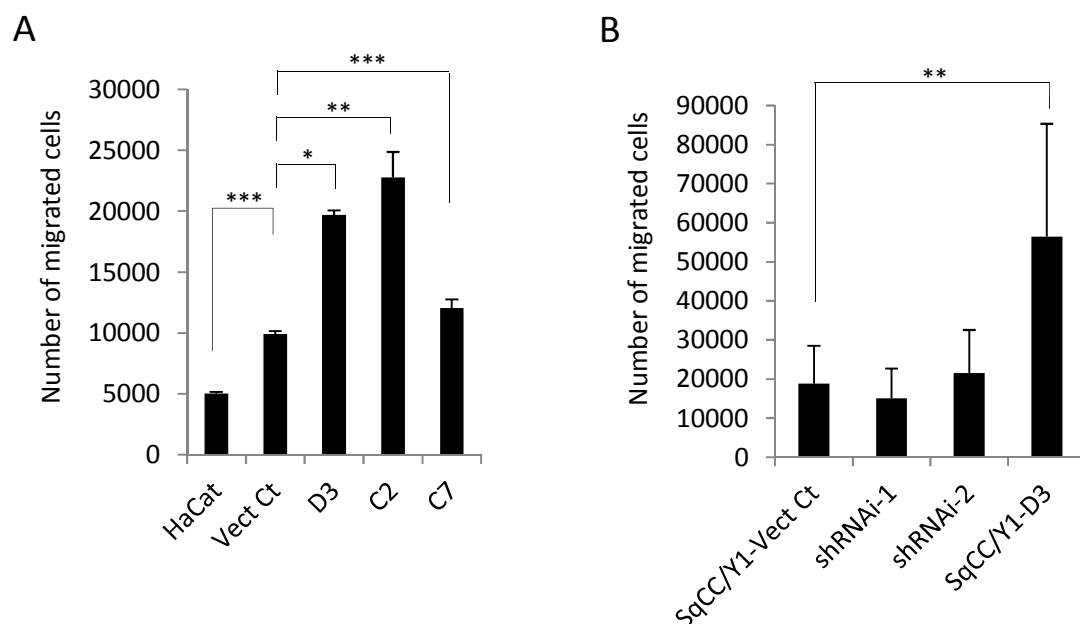


Figure 4.4 Desmoglein 3 promotes transwell cell migration. Various cell lines in two cancer cell types were assessed, including A431 and SqCC/Y1. Two stable SqCC/Y1 lines with transduction of Dsg3 specific shRNAi-1 and -2 were included. Left bar chart shows that overexpression of Dsg3 in A431 cells resulted in a significant increase in cell migration (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). HaCat cells which retain normal cell-cell adhesion was used as negative control in this assay and showed a significantly reduction of cell migration as compared to A431-V cell (*** $p < 0.001$). Similarly, overexpression of Dsg3 in SqCC/Y1 cells also shows a significant increase in cell migration (pooled data from 3 experiments, triplicate in each experiment, mean \pm S.D.) For knock down efficacy in SqCC/Y1 cells see Figure 4.6.

cell free zone (wound) was created using a 96-well Woundmaker™. Cells were washed 3 times with PBS to remove any cellular debris followed by addition of growth media supplemented with 10% serum. Finally, the image lock plates were loaded into the IncuCyte™ which was programmed to acquire an image from each well every 15 minutes for 28 h. Dsg3 overexpressing cell lines displayed an increased rate of wound closure compared to Vect Ct cells (Figure 4.5). In support, Dsg3 silencing in A431-D3 cells, as described previously, reduced the rate of wound closure compared to A431-D3 cells treated with scrambled Ct siRNA (Figure 4.5). Taken together, these results support the notion that Dsg3 regulates cell migration in cancer cell lines (Chen, et al., 2007).

4.2.3 Desmoglein 3 promotes cancer cell invasion

Thus far it has been demonstrated that the overexpression of Dsg3 significantly increases membrane protrusion, cell spreading and migration. These observations prompted the question whether Dsg3 overexpression could enhance cell invasion. To

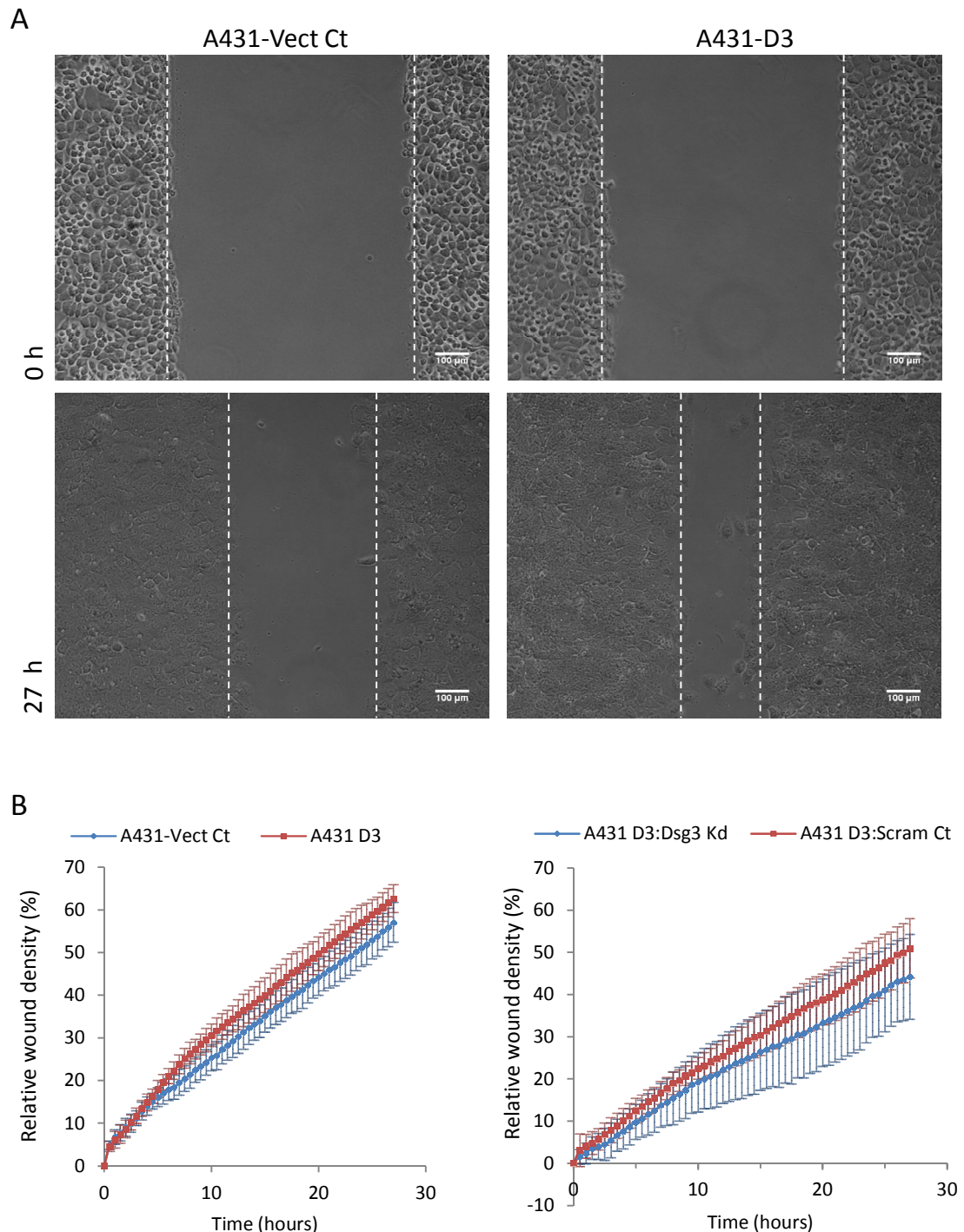


Figure 4.5 Desmoglein 3 promotes two dimensional cell migration. A431 cells either with Dsg3 overexpression or knockdown were seeded at ~80% density in 96-well plate and allowed to form a monolayer overnight. The cell free zones (scratch) was created using a 96-well Woundmaker™ (Essen). The plate was placed into the IncuCyte where images were acquired every 15 minutes over a period of 28 h. Dotted lines represent migrating fronts. **(A)** Illustrates representative images at t 0 and at 27 h for A431-Vect Ct and A431-D3 cells. Dotted lines highlight the invasive fronts. Scale bar 100μm **(B)** Relative Wound Density (RWD) was determined by the IncuCyte software, this metric measures the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point. It was designed to be zero at t=0 and 100% when the cell density inside the wound was the same as the cell density outside the initial wound. Compared to control cells, D3 cells showed higher RWD (left) and in contrast, Dsg3 knockdown cells exhibited a reduced RWD (right).

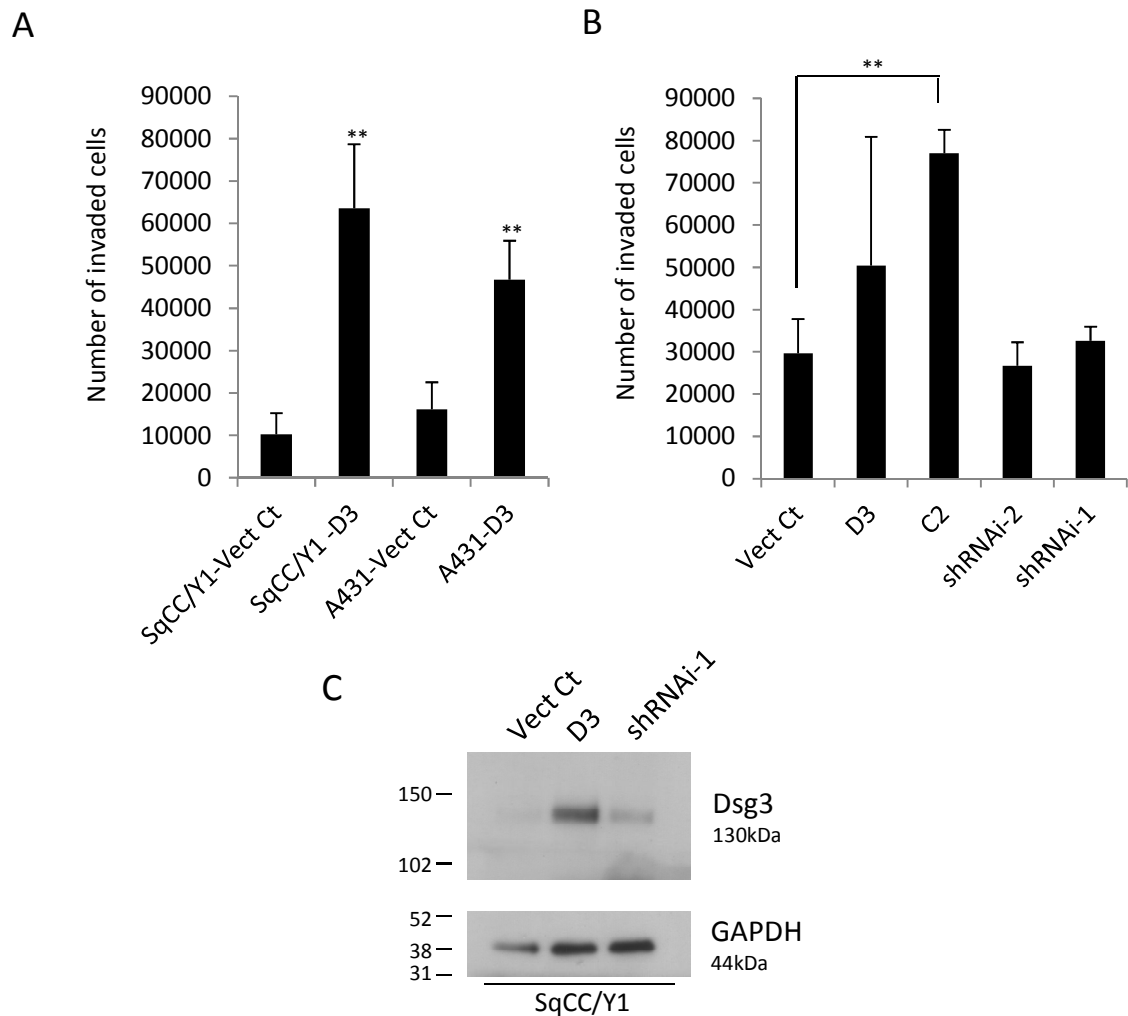


Figure 4.6 Desmoglein 3 promotes cancer transwell cell invasion. (A) Transwell cell invasion of A431 and SqCC/Y1 cell lines was examined and showed consistently that D3 cells displayed 2- or 7-fold increase in cell invasion, respectively. **(B)** Various SqCC/Y1 cell lines along with two stable shRNAi cell lines with Dsg3 knockdown, shRNAi-1 and -2 were also tested (courtesy of Miss Emina Gunic). Only Dsg3 overexpressing cells showed increased cell invasion. In contrast, both knockdown cell lines exhibited similar rate of invasion to that of control cells (** $p > 0.05$). **(C)** Western blot of SqCC/Y1 cell lines to illustrate Dsg3 over expression and silencing efficacy.

test this hypothesis two means of assessing invasion were adopted, the transwell invasion assay and organotypic raft culture, the latter of which is a more physiologically relevant method of assessing cell invasion. The transwell invasion assays were carried out similarly to the above migration assay except here cells are required to invade through a thin layer of Matrigel applied to each insert before the addition of cell suspension. As illustrated in Figure 4.6, a significant increase in cell invasion was observed in A431-D3 (2-fold) and SqCC/Y1-D3 (7-fold) compared to Vect Ct cells (Figure 4.6A). The same observation was made independently by other colleague in the laboratory (Figure 4.6B, courtesy of Miss Emina Gunic). Again, the

Dsg3 knockdown in SqCC/Y1 parental cells had no effect in cell invasion compared to control.

Finally, an organotypic cell invasion assay with Matrigel:collagen gel embedded with primary fibroblasts was performed (courtesy of Miss E Gunic). This assay enables the assessment of cell invasion in a 3-dimensional tissue culture setting that facilitates the full differentiation of a cell monolayer into a Matrigel:collagen gel embedded with primary fibroblasts which mimics physiological condition at the air-liquid interface. Following gel preparation (see methods and materials\)) cultures were maintained for 14 days. The organotypic cultures were then fixed by submerging in Trumps fixative overnight. Specimens were then embedded in paraffin wax and sectioned for staining with haematoxylin and eosin. Histological images were acquired (Figure 4.7A) and analysed to determine invasion index (Figure 4.7C) as previously described (Thomas, et al., 2001). The invasion index was devised by as a means to estimate the cell invasion *in vitro* taking into account the parameters including average depth of invasion, the number and area of invading cell clusters (particles), an example of which is indicated by the red arrow in Figure 4.7A. This index maximises differences in cell invasion and also reflects the cell invasion pattern in the gels. A significantly high invasion index with respect to the control indicates increased cell invasiveness. Analysis of ~13 images in each specimen revealed that D3 cells, compared to the vector control, showed significantly increased invasive depth, number of particles, invasive area and the average size of the particles (Figure 4.7B), which consequently resulted in a 3-fold increase in the invasive index in SqCC/Y1-D3 compared to SqCC/Y1-Vect Ct cells (Figure 4.7C). Immunohistochemistry with anti-Myc tag antibody was also performed to confirm the expression of exogenous hDsg3.myc in SqCC/Y1-D3 cells (Figure 4.7A).

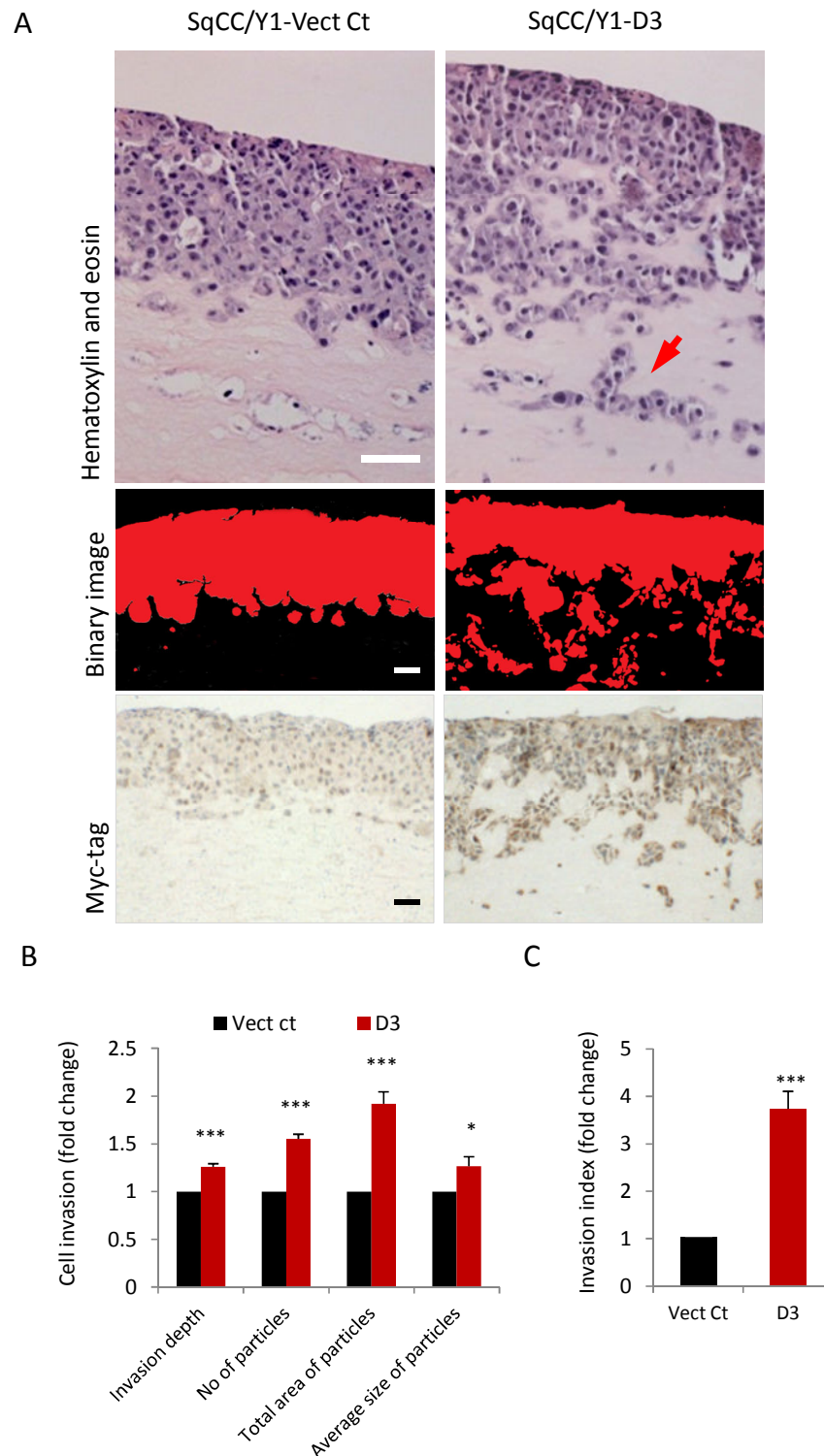


Figure 4.7 Desmoglein 3 promotes three dimensional cell invasion.(A) Hematoxylin and eosin staining of 2-weeks organotypic culture of SqCC/Y1 cells that shows augmented cell invasion in cells with overexpression of Dsg3 (D3, red arrow). The middle panels (Red/Black overlay) are the representative binary images, the epithelial compartment is highlighted in red that enable better visualization of cell invasion. The lower panels are the immunohistochemistry of myc-tag staining demonstrating positive staining of myc-tag in D3 cells (hDsg3.myc transduced cells) but only weak nuclear staining (endogenous c-Myc) in the control sample. (B) Quantification of the cell invasion (pooled from two independent experiments). The invaded epithelial cell clusters were scored for the indicated parameters (n=13, mean \pm SD, *P<0.05, ***P<0.001). (C) cell invasion index that indicated a nearly 4-fold increase in D3 compared to control cells (mean \pm s.e.m., ***P<0.001). Scale bars, 50 μ m.

4.3 Discussion

This chapter provides evidence that Dsg3 promotes cell spreading, membrane protrusions, migration and invasion in cancer cell lines using a variety of techniques. These findings are consistent with previous reports demonstrating a positive role in cancer progression and metastasis (Bancroft, et al., 2002, Chen, et al., 2007, Patel, et al., 2013, Savci-Heijink, et al., 2009).

Dsg3, like other desmosomal cadherins, principally functions in mediating cell-cell adhesion in desmosomes. Logically, one would expect Dsg3 promotes cell-cell adhesion and concomitantly inhibits cell migration when overexpressed (Tselepis, et al., 1998). Paradoxically, the upregulation of Dsg3 was independently reported in SqCCs of the head and neck (Huang, et al., 2010, Patel, et al., 2013), lung (Fukuoka, et al., 2007) and oesophagus (Fang, et al., 2014). Knockdown of Dsg3 in cancer cell lines reduces tumour growth and invasion (Chen, et al., 2013, Chen, et al., 2007). Furthermore, the level of Dsg3 expression has been shown to correlate with the T stage, N stage and overall stage of malignant disease, tumour depth and extra-capsular spread in lymph nodes (Chen, et al., 2007, Ferris, et al., 2005, Patel, et al., 2013). These reports collectively support the participation of Dsg3 in cancer progression. However, a majority of these studies are based on genetic profiling apart from one study which investigated, mechanistically, the consequence of *DSG3* knockdown in head and neck cancer cell lines (Chen, et al., 2007). Studies using a controlled model system with respect to Dsg3 expression is still lacking, in particular a gain-of-function model with ectopic Dsg3 overexpression. This study provides the first attempt of such an experimental approach. Thus the findings from this study further implicate Dsg3 in the promotion of cancer cell migration and invasion supporting it's potential as a potential therapeutic target in preventing cancer progression and metastasis, as proposed by others (Chen, et al., 2007).

Recently, a study examining the role of Dsg3 in carcinogenesis based on the Dsg3 knockout mouse model has been carried out (Baron, et al., 2012). Using two models of skin carcinogenesis, they concluded that the suppression of Dsg3 does not inhibit carcinogenesis. In the first approach, they examined tumourigenicity potential of transformed Dsg3^{-/-} and Dsg3^{+/-} keratinocytes in immune-compromised *scid* mice and

showed a 20% reduction in tumour development and a reduction in tumour volume in mice injected with Dsg3^{-/-} keratinocytes compared with those infected with Dsg3^{+/-} keratinocytes, suggesting that Dsg3 may facilitate tumour growth in certain settings. Their second approach examined UVB-induced SCC development in Dsg3^{-/-} mice compared to wild type mice. This study revealed no significant difference in tumour latency, size and multiplicity between the two cohorts suggesting that Dsg3 deficiency does not promote nor inhibit tumorigenesis in the epidermis upon UVB exposure (Baron, et al., 2012). Another study carried out by Garrod and colleagues in which Dsg3 was mis-expressed in the epidermis of transgenic mice driven by the K1 promoter. They found that these mice exhibited hyperproliferation, abnormal differentiation and parakeratosis of the epidermis but no evidence of tumour development was reported in this Dsg3 overexpression model (Merritt, et al., 2002). Parakeratosis describes the incomplete maturation of epidermal keratinocytes resulting in abnormal retention of nuclei and this condition is seen in diseases associated with increased cell turnover including SCC. While these studies are not in keeping with the data presented, they suggest that Dsg3 may not function as a main driver in tumorigenesis. However what these studies do not exclude is the role of Dsg3 in cancer progression and metastasis, where aberrant Dsg3 expression has been identified and suggested to participate (Chen, et al., 2007, Gordon, et al., 2003, Patel, et al., 2013, Savci-Heijink, et al., 2009, Wang, et al., 2007).

In agreement with my proposal, a recently publication reported that Dsg3 regulates Rac1/Cdc42 to modulate actin organisation and dynamics (Tsang, et al., 2012). Rac1 and Cdc42 belong to the family of small Rho GTPases and, upon activation they confer the formation of lamellipodia and filopodia, respectively (Tsang, et al., 2012). Activation of Rac1 and Cdc42 has long been known to be associated with tumour cell invasion and metastasis (Ma, et al., 2013, Zins, et al., 2013). These findings support the observations of cell spreading and membrane protrusion in association with Dsg3 expression and are also consistent with previous reports (Tsang, et al., 2010).

Taken together, the specific role of Dsg3 in cancer is still open to interpretation. Thus far studies suggest Dsg3 overexpression is not tumourigenic. However its overexpression promotes migratory and invasive behaviour in cancer cells and its inhibition reduce invasive behaviour *in vivo*. Together with the clinical correlation

between Dsg3 expression cancer stage and detection in metastatic lymph nodes support its role in cancer progression and metastasis.

4.4 Future work

To develop this aspect of the project the use of animal models would undoubtedly provide invaluable information as to the role of Dsg3 in tumorigenesis. In the few studies that have overexpressed Dsg3 *in vivo* (Elias, et al., 2001, Merritt, et al., 2002) neither have looked at the oncogenic potential of Dsg3 in these scenarios, however in both cases precancerous events including decreased cell-cell adhesion and hyperproliferation respectively were observed. One question that remains is whether Dsg3 has the ability to transform cells or whether it is a latter participant in the progression of disease. To address this question analysis of Dsg3 overexpression in normal cells would provide information on the transforming ability of Dsg3. Additionally, using transgenic mouse models engineered for the targeted conditional knockout and overexpression of Dsg3 by using the Cre-Lox recombination system for example which would allow Dsg3 targeting without disrupting it's essential function in epithelial junctions and tissue integrity. The understanding yielded from such projects with regards to Dsg3s participation in the development and/or progression of cancer would justify the use of Dsg3 as a potential therapeutic target. Currently there is no definitive evidence as to its precise role in cancer, however its inhibition in cancer cell lines and Xenograft models identifying it as an effective therapeutic target. Its use as a biomarker for lymph node metastasis highlights that Dsg3 should not be ignored. If I were to speculate, how Dsg3 interacts with and subsequent signalling events arising from the tumour environment may provide the answer by providing key understanding as the precise role of Dsg3 in the progression of cancers which may yield a comprehensive understanding of Dsg3's role in oncogenesis. To compliment the above a prognostic study could determine is Dsg3 over expression has any prognostic value by look at survival in a cohort of patients with early stage SqCC of the head and neck and lung.

The techniques used to examine morphology in this study were largely qualitative with which could arguably be considered subjective and open to bias. In future, by employing automated tool such as FiloDect (Nilufar, et al., 2013) for quantification of filopodia in microscopy images and a quantitative metrics approach to characterise other morphological characteristics would be strengthen the data presented.

Another consideration is the potential contribution of cell proliferation on the outcome of migration and invasion analysis where mitomycin C was omitted, specifically in the scratch assay assessed using IncuCyte, transwell and organotypic assays. While the time periods over which the scratch and transwell assays were limited to 24-48 h, would limit proliferative affect without the direct assessment of proliferation in both Vect Ct and –D3 cells its potential contribution to the migration front in the scratch assays and the number of migrated cells in the transwell assays cannot be ignored particularly as the aberrant expression of Dsg3 *in vivo* and *in vitro* has been reported to affect cell proliferation (Chen, et al., 2007, Merritt, et al., 2002). However, despite the potential proliferative contribution the results from experiments without mitomycin C supports those carried out in its presence, suggesting that in the cell systems used the proliferative affect is small and possible negligible. Again a definitive conclusion can only be attended upon investigation.

Finally, the fibroblasts in the organotypic cultures are difficult to identify in the gels. Staining using fibroblast markers such as type III Intermediate filament protein, Vimentin, which is frequently found in fibroblasts and fibroblast surface antigen (SFA) a glycoprotein produced by fibroblasts.

Chapter 5: **Desmoglein 3 colocalises and forms a complex with Ezrin.**

5.1 Introduction

Desmoglein 3 has been shown to participate in a multitude of cellular functions beyond mediating cell-cell adhesion as outlined in chapter one. In particular, it has been implicated in promoting membrane dynamics and changes in cell morphology that are associated with cell migration, invasion and metastasis consistent with the evidence presented in the previous chapter. However, how Dsg3 is able to achieve these functions is not fully understood.

Cell morphogenesis, migration and invasion are cellular processes to which the actin cytoskeleton is essential as it provides the driving force behind membrane dynamics and plasticity to initiate and facilitate these highly integrated and multistep processes. In pathological situations, such as tumour cell migration and invasion, which evoke these actin-based cellular processes, modulation of actin and its regulators is essential. Dsg3-dependent regulation of actin organisation has been suggested previously in mechanistic studies of PV, which showed that binding of auto-antibodies to the keratinocyte provokes cortical actin reorganisation (Gliem, et al., 2010, Waschke, et al., 2006). In support, a recent study has demonstrated that Dsg3 is required for peripheral

cortical actin assembly (Tsang, et al., 2012) Another independent study showed that desmosome assembly and dynamics in the process of epithelial cell migration requires the actin cytoskeleton (Roberts, et al., 2011). Collectively, these reports suggest crosstalk between Dsg3 and the actin cytoskeleton and that desmosomes and their constitutive proteins, including Dsg3, are capable of modulating actin and its associated regulators to facilitate membrane dynamics and cell migration. This crosstalk may provide a mechanism by which Dsg3 regulates cell migration and invasion. This proposal is supported by the implication of Rho GTPases as downstream effectors of Dsg3-dependent actin organisation (Spindler and Waschke, 2011, Tsang, et al., 2012, Waschke, et al., 2006).

Actin based processes such as membrane dynamics, cell motility and invasion require orchestration and regulation. The ERM (Ezrin, Radixin and Moesin) family proteins are identified as playing a key role in these processes through their cross-linking activity. The ERM proteins are concentrated at cell-surface structures, such as microvilli, filopodia, uropods, ruffling membranes and cell adhesion sites where actin is associated with the plasma membrane (Amieva and Furthmayr, 1995, Bretscher, 1999, Sato, et al., 1991, Serrador, et al., 1997). It has been shown that silencing of ERM proteins using antisense oligonucleotides inhibits microvillus formation and cell adhesion (Takeuchi, et al., 1994) and perturbs membrane ruffling and cell motility (Lamb, et al., 1997, Paglini, et al., 1998). Precisely how ERM proteins regulate the formation and/or maintenance of these structures remains unclear. However, there is evidence to suggest that the ERM proteins not only directly link the actin cytoskeleton to the plasma membrane but also may exert orchestrative functions through multi-protein complex assembly in specific cellular compartments (Arpin, et al., 2011) (see section 1.3.3 Function of ERM proteins). The ERM protein, Ezrin, has been shown to be dynamically regulated during cancer progression, with reports of hyper-phosphorylation (activation) and increased expression levels in a variety of cancers (Chen, et al., 2011, Chuan, et al., 2006, Huang, et al., 2011, Khanna, et al., 2004, Ma and Jiang, 2013, Meng, et al., 2010, Ren, et al., 2009, Saito, et al., 2013, Xie, et al., 2011).

The colocalisation between Dsg3 and Ezrin in cancer cells was initially demonstrated by Dr Wan (Tsang, et al., 2010). The characterisation of such an association and the

nature of their interaction could provide a novel mechanism for Dsg3 in regulating tumour cell morphology, migration and invasion and further our understanding of the role of Dsg3 in cancer progression and metastasis. This Chapter aims to characterise the association between Dsg3 and Ezrin using a combination of microscopy and biochemical techniques.

5.2 Results

5.2.1 Desmoglein 3 colocalises with Ezrin predominantly at the basal domain of the plasma membrane.

The preliminary analysis revealed that Dsg3 colocalises with Ezrin, particularly at protrusions of the plasma membrane (Figure 1.X) (Tsang, et al., 2010). To validate this finding, confocal and epi-fluorescence microscopy was performed on A431-D3 and A431-Vect Ct cells grown on coverslips for 24 h (n=10). The following day cells were fixed and stained using Dsg3 (5H10) and Ezrin specific antibodies. At least, 3 random fields from each coverslip were acquired. Figure 5.1A illustrates representative images of Dsg3 and Ezrin staining, as shown there is a clear over expression of Dsg3 in A431-D3 cells compared to the Vect Ct as well as a high level of colocalisation (represented by the yellow/orange pixels). Analysis of the observed colocalisation using ImageJ to determine average percentage colocalisation per cell revealed a significant ($p<0.01$) six-fold increase in the average percentage colocalisation in A431-D3 cells compared to A431-Vect Ct. In support knockdown of Dsg3 in A431-D3 cells reduced Dsg3:Ezrin colocalisation (Figure 5.1B), suggesting the observed colocalisation is Dsg3-dependent. In support analysis of percentage colocalisation per cell revealed a significant ($p<0.01$) reduction in Dsg3:Ezrin colocalisation in knock down cells compared to the scrambled Ct. This analysis was expanded to include Dsg3, A431-C11 (low expression) and C2, C6 and C7 (high expression) to reveal a similar trend (Figure 5.2) with a clear correlation between Dsg3 relative expression and Dsg3:Ezrin colocalisation, represented by the yellow/orange pixels. ImageJ analysis of colocalisation with respect to Dsg3 expression revealed a positive correlation. Finally the overexpression of Dsg3 appeared to have no significant effect on the total ezrin expression (Figure 5.2). Similarly the colocalisation between Dsg3 and pERM in cells with high Dsg3 expression (A431-D3, C2, C6 and C7), exhibited high levels of colocalisation in the basal planes compared to the vector control and low expression clone C11 (Figure 5.3B).

5.2.2 Desmoglein 3 colocalises with Ezrin in close proximity.

The above analysis of the Dsg3:Ezrin colocalisation was based on the qualitative assessment and quantification of overlapping pixels for both channels as measured by ImageJ, expressed as percentage colocalisation. However, the threshold for

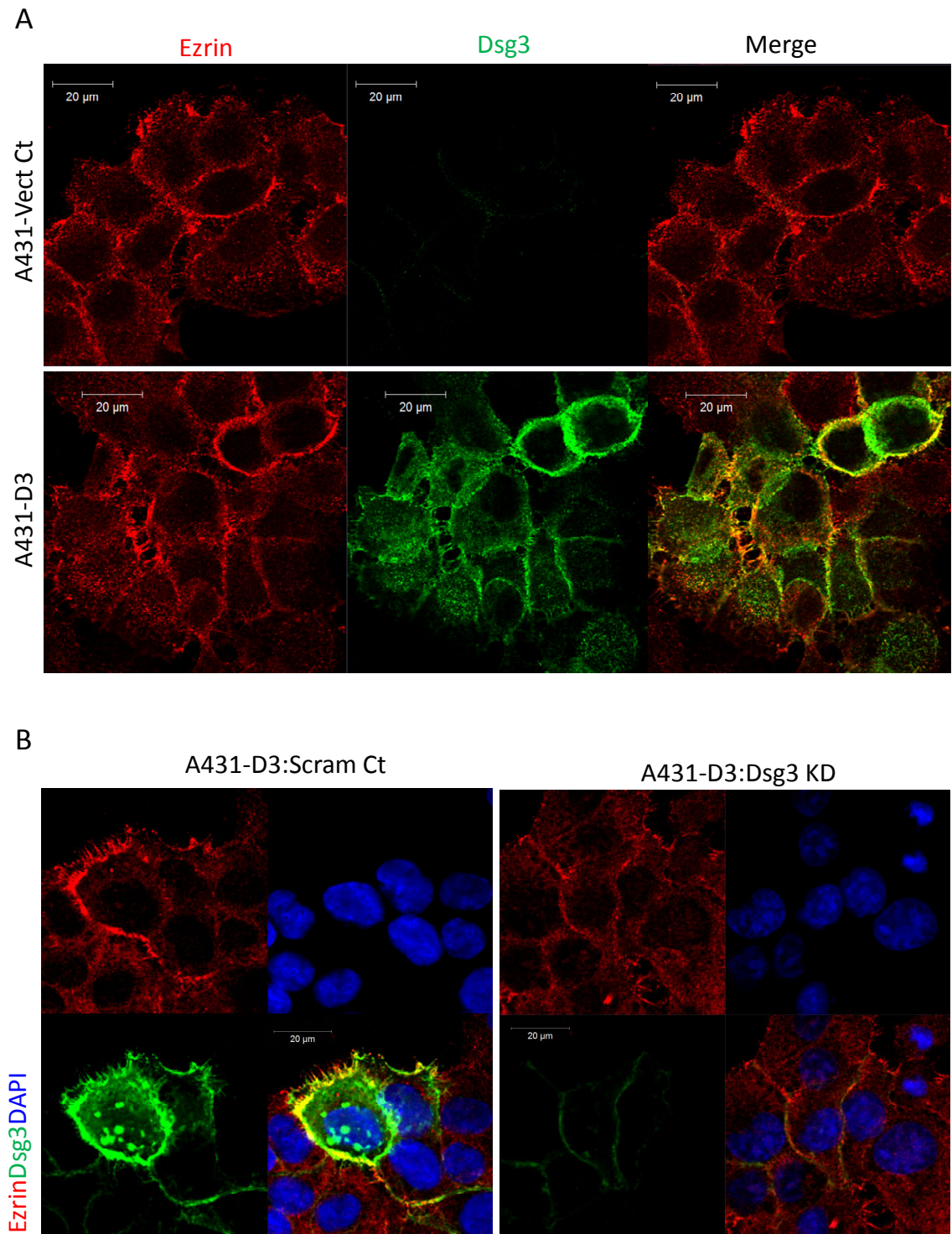


Figure 5.1. Desmoglein 3 colocalises with Ezrin. (A) A431 cell lines of vector control (A431-Vect Ct) and Dsg3 overexpressing D3 cells were seeded on coverslips at 50-60 % confluence prior to fixation 24 h later. Cells were fixed with ice cold Methanol:Acetone (1:1) before immunostained for Dsg3 and Ezrin.(n=10) (B) In the knockdown experiment, A431-D3 cells were transiently transfected with either Dsg3 specific or scrambled control siRNA. Cells were fixed and immunostained 48 h after transfection (n=5). In each experiment at least three fields per coverslip were imaged using confocal or epi-fluorescence microscopy. Representative images are shown in this figure Scale bars, 20 μ m.

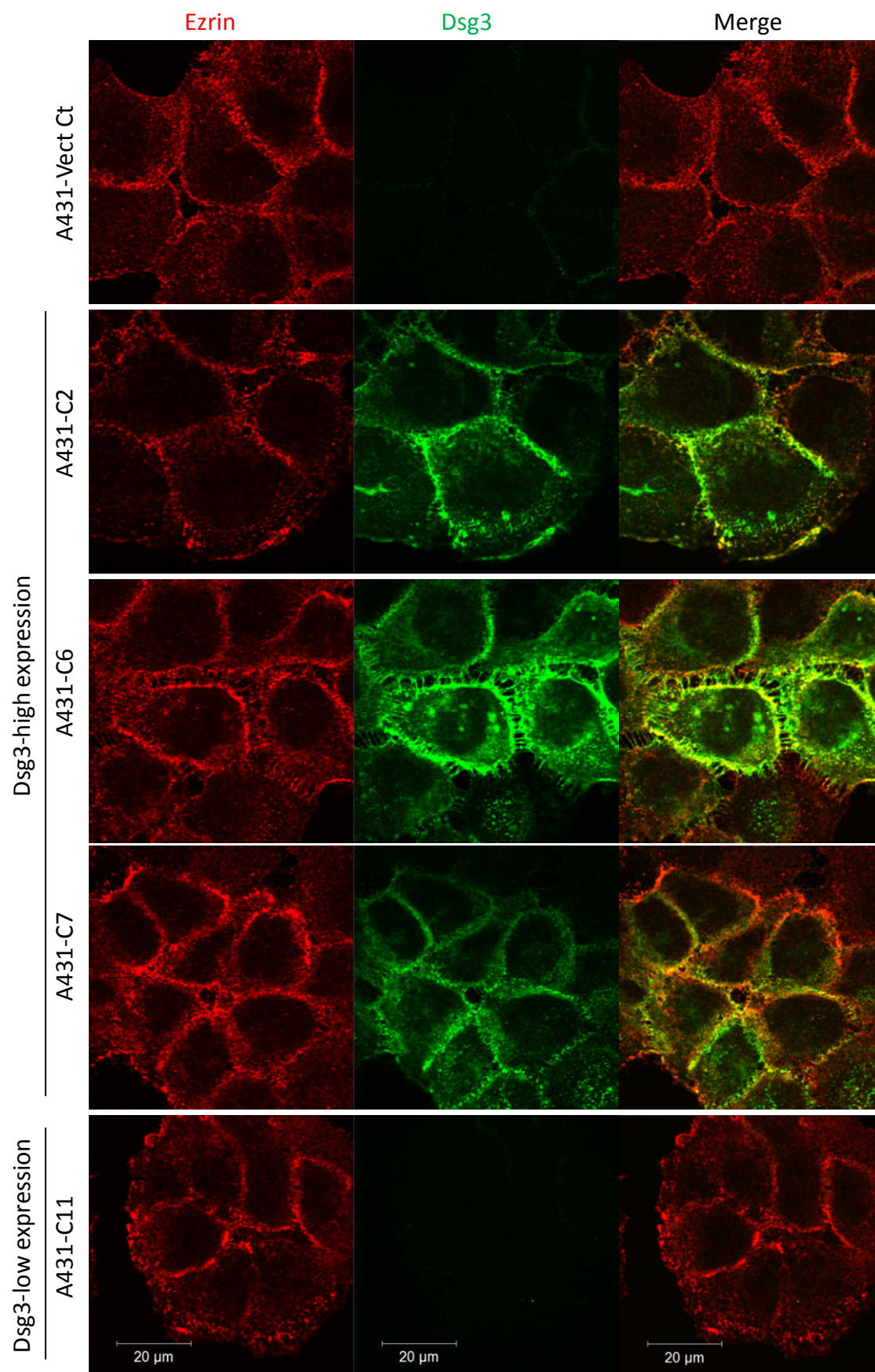


Figure 5.2. Desmoglein 3 colocalises with Ezrin in a dose-dependent manner. (A) A431-Vect Ct, -C2, -C6, -C7 (Dsg3 high) and -C11 (Dsg3 low) were double immunostained for Dsg3 and Ezrin 24 h after being seeded at 50-60% confluence. Colocalisation between Dsg3 and Ezrin appear as yell/orange pixels (n=3. Scale bar, 20 μ m)

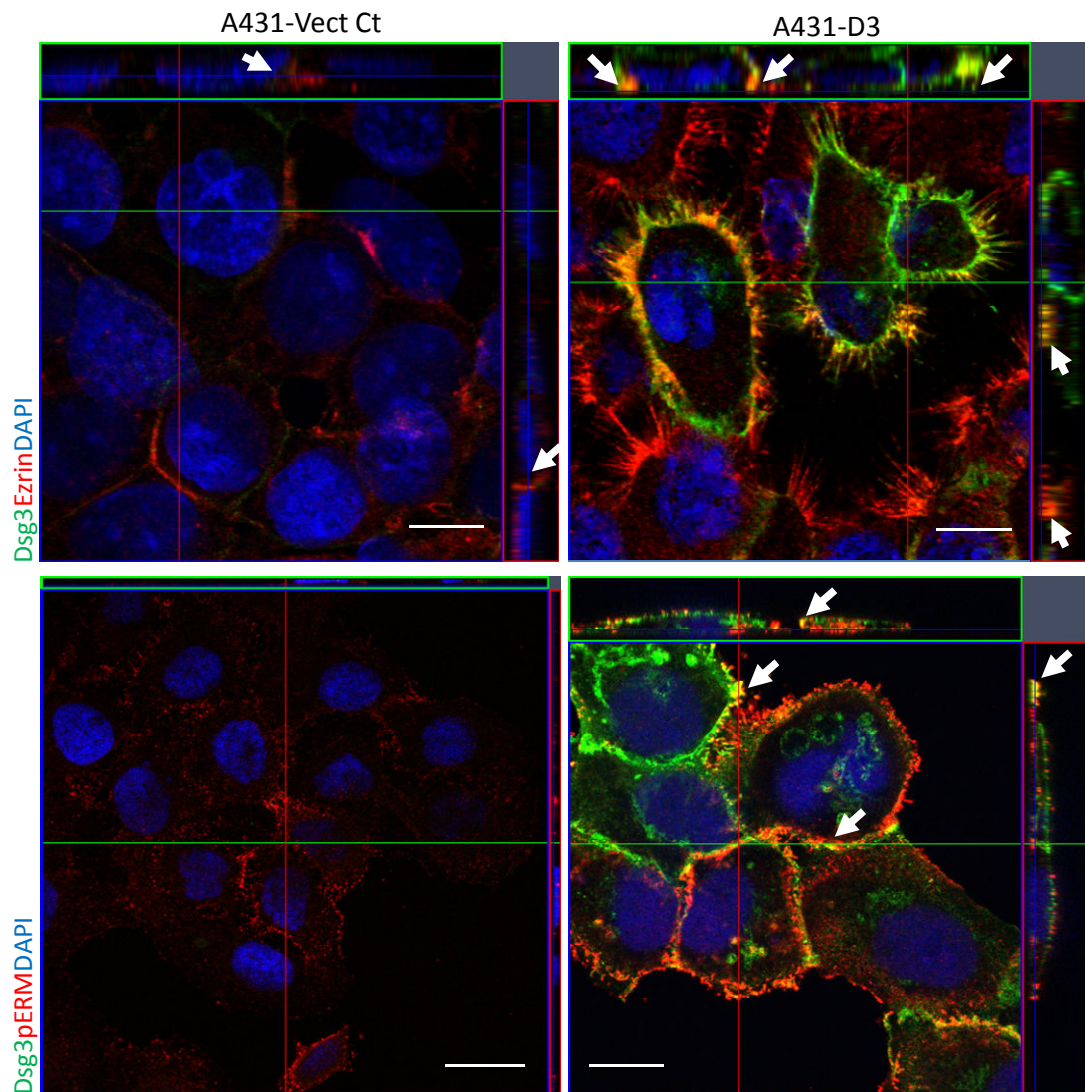


Figure 5.3. Desmoglein 3 colocalises with Ezrin at the basal domain of the plasma membrane. Confocal Z stacks of A431-Vect Ct and –D3 cells double stained for Dsg3 and Ezrin (Top: Z Stack: 1 μ m intervals. total Z distance: 6 μ m) or pERM (bottom: Z stack V and D3 cells at intervals: 0.29 μ m and 0.34 μ m respectively. Total distance: 2.29 μ m and 12.07 μ m, respectively). Colocalisation between Dsg3 with Ezrin and pERM is represented by yellow/orange pixels. Colocalisation of Dsg3 with Ezrin and pERM appears to be concentrated at the plasma membrane where membrane projections are predominant and in the basal plans the of the Z stacks (white arrows). Scale bars, 20 μ m.

colocalisation of fluorescence using epi fluorescence and confocal microscopy between 400-600 nm. As a result it remained unclear how closely these two molecules were associated at the plasma membrane. To further examine the intimate relationship between Dsg3 and Ezrin several techniques were employed. Firstly, the Proximity Ligation Assay (PLA) was performed which allows for the identification and quantitation of molecular interactions within 10-40 nm (see section 2.4.6 Proximity ligation assay). Representative images of this analysis are illustrated in Figure 5.4 A-C

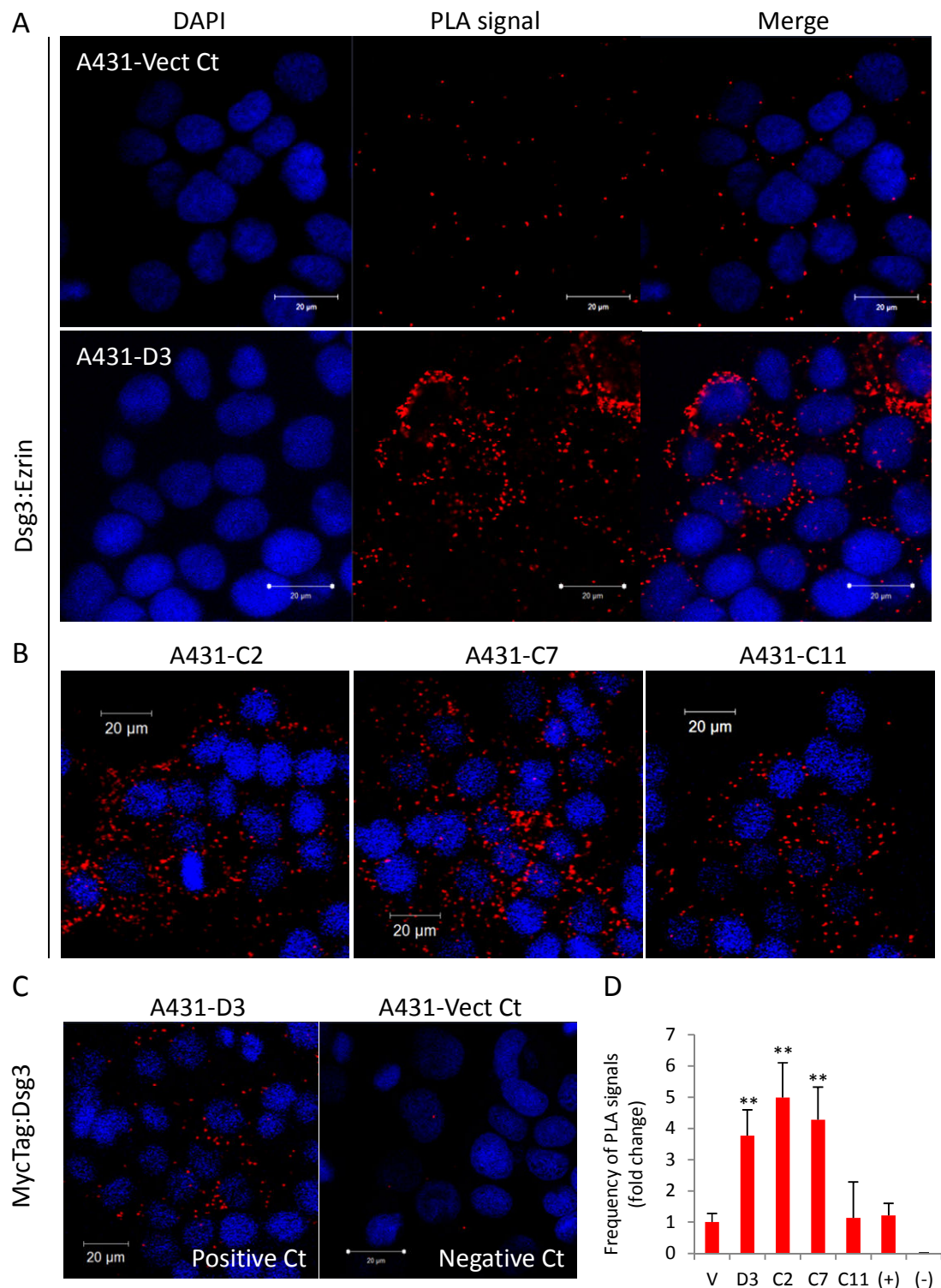


Figure 5.4. Desmoglein 3 and Ezrin associate between 10-40 nm in a Dsg3-dose dependent nature. A431 cells seeded at 50-60% density followed by fixation with Methanol:Acetone (1:1). The assay was carried out according to manufacturers guidelines, using blocking buffers, incubation times and antibody concentrations optimised for immunofluorescence protocols. Images were acquired using confocal microscopy and analysed using ImageJ. (n=2, 3 fields per cell line were analysed. **(A, B)** show Dsg3 and Ezrin interactions within 10-40 nm, each interaction is represented by a single red signal. **(C)** Positive and negative Ct, here signal represent interactions between Dsg3 and MycTag in A431-D3 and -Vect Ct cells respectively. **(D)** Quantitation of interactions frequency per cell, presented as Mean fold change \pm SD., **P < 0.01. Scale bar 20 μ m.

(n=2) which shows a 3.5, 5 and 4-fold increase in the frequency of interactions (Figure 5.4 C) between Dsg3 and Ezrin in A431-D3, C2 and C7 cells, respectively, compared to Vector Ct cells. No difference was seen between C11 and control cells.

Secondly, FRET (Fluorescence Resonance Energy Transfer) analysed by acceptor photobleaching was performed to determine if Dsg3 and Ezrin were interacting within 10 nm (see section 2.4.5 Fret by acceptor photobleaching). For this analysis, A431-D3 and Vect Ct cells were stained with Dsg3 and Ezrin specific antibodies. The secondary antibody pair in this case was anti-mouse IgG conjugated with Alexa Flour 488 for Dsg3 and anti-rabbit IgG with Alexa Flour 555 for Ezrin labelling. FRET analysis was performed on numerous cellular regions, including cell-cell contacts, membrane protrusion and cytoplasm of A431-Vect Ct and-D3 cells alongside the acquisition of controls. Altogether, 89 potential FRET regions were analysed. Figure 5.5 A, B illustrates representative examples of pre- and post-bleaching images with test and control regions identified alongside relative integrated density of the donor and acceptor fluorescence. Positive FRET was detected and exhibited as an increase in donor fluorescence (Region 1 and 2 in the Dsg3 post-bleach images in Figure 5.5A. Of the 89 regions analysed, 13 (14%) displayed positive FRET above the threshold of 5%, with an average FRET efficiency of $8\% \pm SD$ (5.5 C), these regions were predominantly located at membrane protrusions.

Finally, further quantification of the Dsg3:Ezrin colocalisation was performed in order to establish that the percentage colocalisation obtained in Figure 5.1-5.3, which was based on the assessment of overlapping pixels, were of equal significance in both A431-Vect Ct and -D3 cells. This was necessary to establish due to the vast difference in Dsg3 expression which may skew the qualitative outcome. Using ImageJ's Intensity Correlation Analysis plug-in which employs a number of coefficients, including 1) *Pearson's Coefficient* which determines the strength of the linear relationship between two channel intensities and is not influenced by background or image brightness. This Coefficient yields values ranging from +1 for perfect colocalisation to -1 for perfect exclusion with a value of 0 occurring due to random localisation; 2) *Mander's Coefficient* assesses the fraction of pixels that overlap and is not influenced by the differences in absolute signal intensities. *Mander's Coefficient* ranges from 0 for no colocalisation and 1 for complete colocalisation; and 3) *Intensity Correlation Quotient*

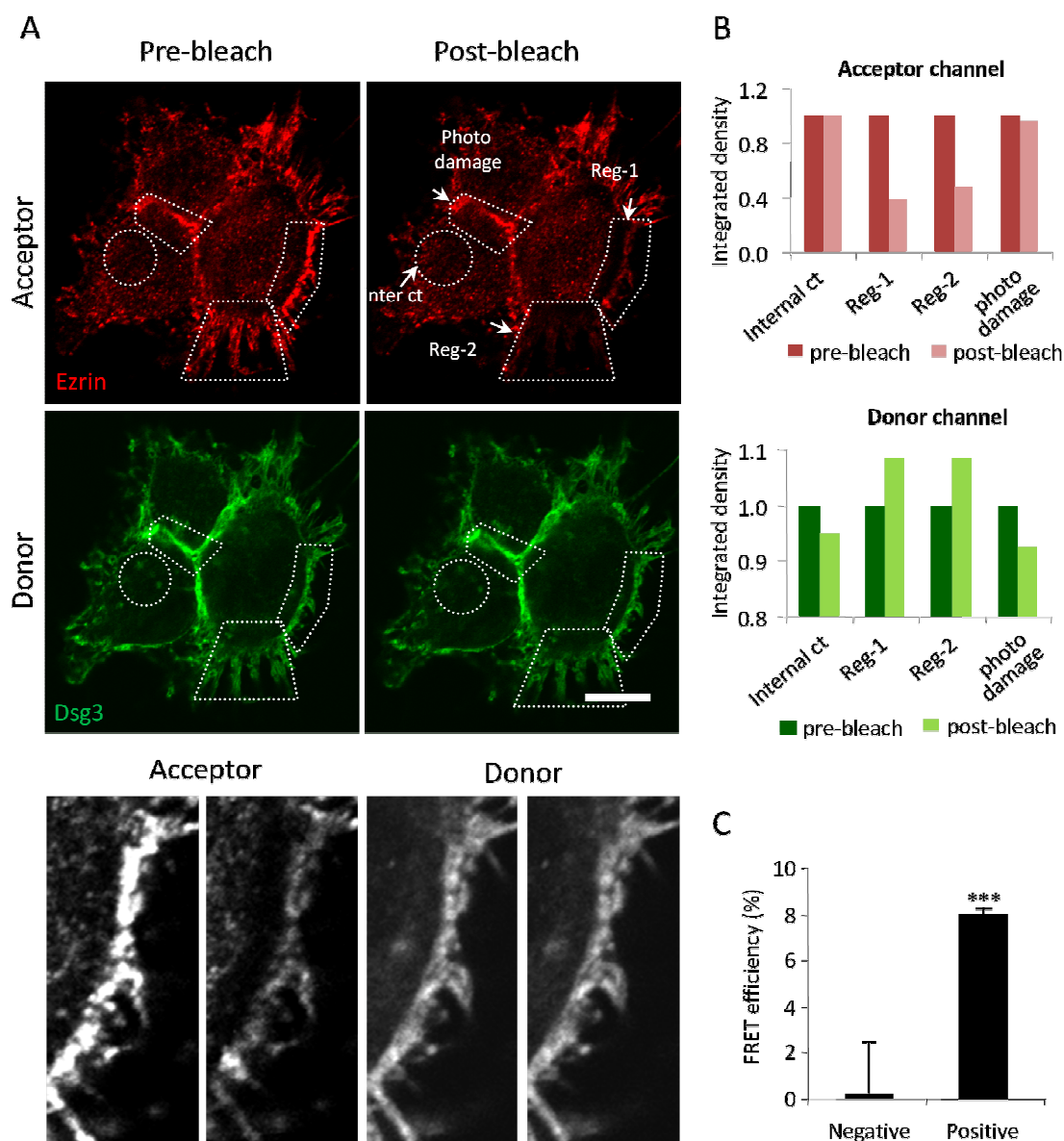


Figure 5.5. Desmoglein 3 and Ezrin associate with 10nm and the plasma membrane. FRET by acceptor photobleaching analysis using confocal microscopy: A431 D3 cell lines were seeded at 50-60% confluence 24 h before fixation with Methanol:Acetone (1:1). Dsg3 and Ezrin were detected using Ezrin and Dsg3 primary antibodies followed by the secondary FRET pair ab FITC and TRITC. The figure illustrates a representative region that was analysed with donor and acceptor channel displayed pre and post photobleaching in addition to enlarges images of highlighting region 1 (reg 1). Photo-damage of approximately 60% was achieved during photobleaching. Fluorescent intensities of all highlighted regions are quantified graphically in the right hand panel. Among 89 regions which include membrane protrusion and intracellular junctions 13 (~14%) displayed positive FRET efficiency above a threshold of 5% with an average FRET efficiency of ~8% (C).

(ICQ) which assesses the synchrony of two signals by analysing the Product of the Difference from the Mean (PDMs). Synchronous intensities result in positive PDMs, whereas asynchronous intensities give rise to negative PDMs. ICQ values are distributed between 0.5 and -0.5 where random staining = 0, segregated or asynchronous staining = 0 > ICQ ≥ -0.5, dependent or synchronous staining = 0 < ICQ ≤ +

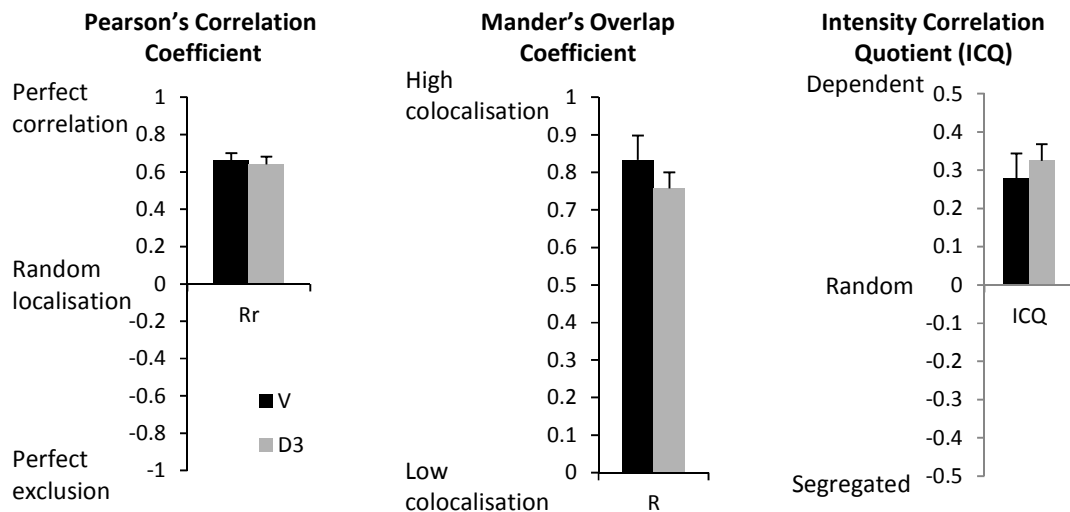


Figure 5.6 Colocalisation between endogenous and exogenous Dsg3 with Ezrin is equally significant in A431-Vect Ct and A431-D3 cells. The colocalisation parameters were determined with ImageJ plug-in, such as Pearson's correlation Coefficients, Mander's Overlap Coefficients and 9 Correlation Quotient for Dsg3 and Ezrin staining and showed the colocalisation in both cells types was of equal significance (Data presented are the coefficient value \pm SD, n=2)

0.5. All these coefficients have an advantage over percentage colocalisation as they analyse all pixels based on their intensity rather than pixel 'overlapping'. As shown in Figure 5.6, all the coefficient analyses supports the qualitative percentage colocalisation for both endogenous and exogenous Dsg3 with Ezrin in A431-Vect Ct and A431-D3 cells with coefficient values consistent with near perfect correlation, *i.e.* *Pearson correlation Coefficient*: A431-Vect Ct, ~ 0.6 and A431-D3, 0.6 ; *Mander's Overlap Coefficient*: A431-Vect Ct, ~ 0.8 and A431-D3, ~ 0.75 ; and *ICQ*: A431 Vect Ct, ~ 0.25 and A431-D3, 0.3 (Figure 5.6). Taken together, the data suggest that Dsg3 and Ezrin are in close proximity with interactions occurring within ~ 10 nm particularly at the membrane protrusions located at the basal domains of the plasma membrane.

5.2.3 Desmoglein 3 forms a molecular complex with Ezrin and possibly Radixin.

Given the above observation suggesting a close association between Dsg3 and Ezrin, it seemed possible that these two proteins may physically interact with each other in a molecular complex. To address this question, co-immunoprecipitation (co-IP) assay of lysates from A431-Vect Ct and -D3 cells were performed. Cells were grown at 50-60% confluence for 24 h, cell lysates were then extracted with RIPA buffer (Materials and Methods). One mg of total protein per sample was used for co-IP with Ezrin antibody (rabbit) and isolated immunoprecipitates were analysed by Western blot. The representative results from six independent experiments are shown in Figure 5.7A

Dsg3-dose-dependent, this study was expanded to include A431-C7 and -C11 cells, further demonstrated a correlation between Dsg3 expression and the amount of associated with Ezrin (Figure 5.7B).

In parallel, the reverse approach using Dsg3-specific antibodies, probing for Ezrin was performed (n=3), no detectable association of these two proteins was observed. However co-IP assay using *mycTag* specific antibody resulted in the identification of a discrete band at the molecular weights similar to that of Ezrin and other ERM proteins (data not shown). However this assay required further optimisation in order to draw a definitive conclusion.

There is evidence suggesting functional redundancy amidst the ERM family proteins, which raised the question whether Dsg3 could potentially associate and possibly form a complex with the other ERM proteins, Radixin and Moesin. To address this question, immunofluorescent microscopy was carried out on A431-Vect Ct, -D3, -C7 and -C11 cells. As illustrated in Figure 5.8, Dsg3 exhibited a predominant plasma membrane localisation, a pattern similar to that of Ezrin. Radixin displayed a predominantly diffused cytoplasmic staining with some membrane association while Moesin is localised heavily in the cytoplasm with strong nuclear staining and respectively exhibits the least plasma membrane localisation (Figure 5.8A). Analysis of immunostaining profiles generated from fluorescent micrographs clearly indicated that Ezrin but not Radixin and Moesin shares similar cellular distribution to Dsg3 (Figure 5.8B). To examine whether Dsg3 could also form a complex with Radixin or Moesin, co-IP with Radixin and Moesin antibody was performed (n=3). The results suggest that Dsg3 might form a complex with Radixin but not Moesin (Figure 5.7C).

5.2.4 Non-junctional Dsg3 colocalises and forms a complex with Ezrin.

Two pools of Dsg3 proteins are found to be present in epithelial cells, characterised as detergent soluble and insoluble, these pools have been shown to be non-junctional and junctional, respectively, the latter of is being associated with desmosomes (Aoyama and Kitajima, 1999, Jennings, et al., 2011, Yamamoto, et al., 2007). Although the function of junctional Dsg3 in cell-cell adhesion has been characterised it remains unclear whether Triton X-100 soluble, non-junctional Dsg3, has additional roles beyond

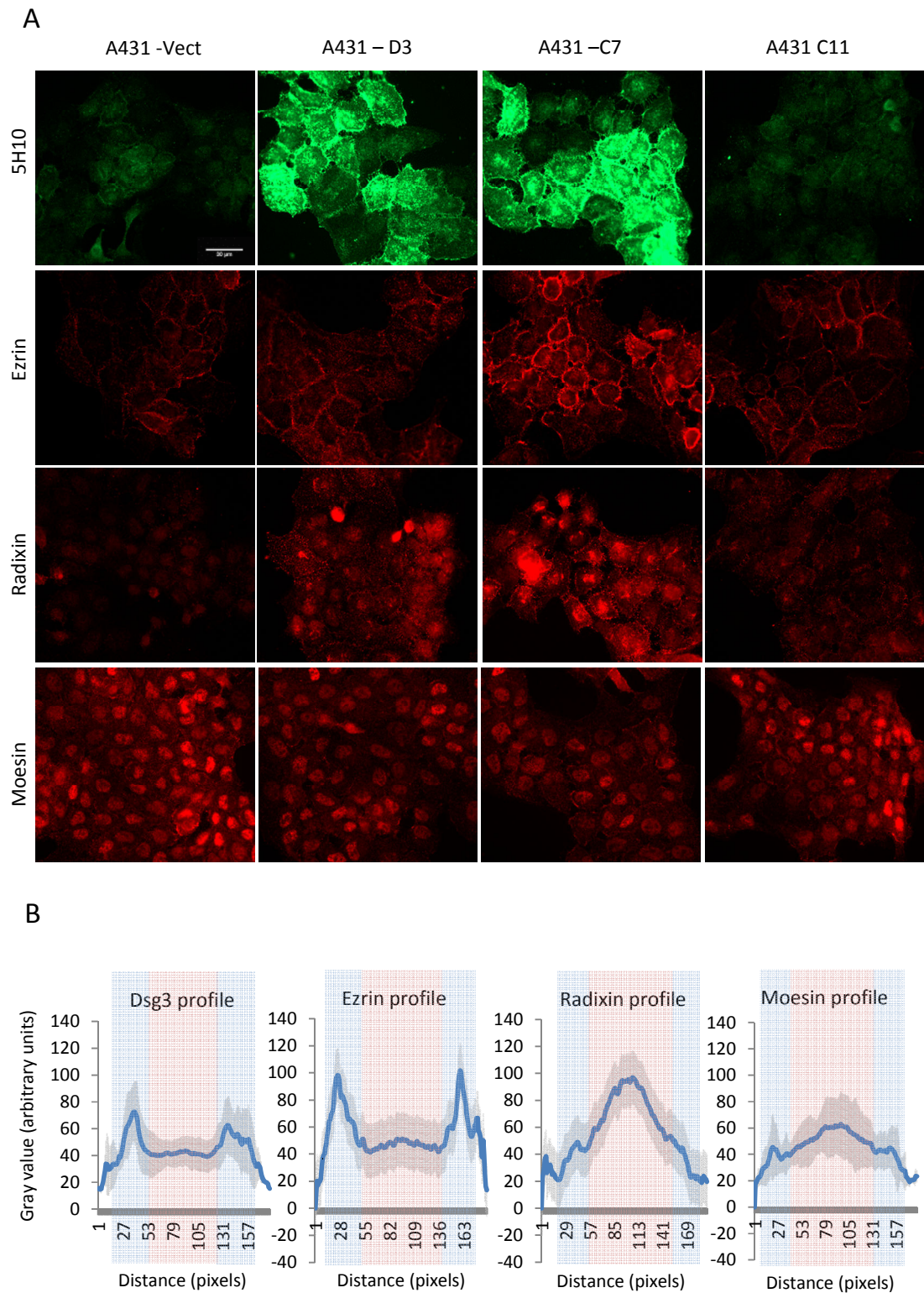


Figure 5.8 Ezrin but not Radixin and Moesin colocalises with Dsg3 at the plasma membrane. **(A)** 24 h after seeding A431-D3 cells at 60-70% confluence on coverslips, cells were fixed and immunostained for Dsg3, Ezrin, Radixin and Moesin. Three images per coverslip were obtained, from which representative images are displayed (Exp n=2). Scale bar, 20µm. **(B)** Staining intensity profiles acquired using ImageJ. Seventy-five cells in total were analysed to determine the cellular localisation of Ezrin, Radixin and Moesin. Data presented are Mean Gray Value \pm SD.

acting as precursors for desmosome assembly and turnover (Green, et al., 2010). Recent studies carried out by Dr Wan's group suggest that non-junctional Dsg3 is involved in regulating Src activation and actin dynamics (Tsang, et al., 2012, Tsang, et al., 2012, Tsang, et al., 2010). Thus, it was hypothesised that non-junctional Dsg3 may possess a function in signal transduction and actin organisation through associations with Ezrin. To address this question, the colocalisation of Dsg3 with Ezrin was analysed in cells where detergent soluble proteins were extracted with Triton X-100 before fixation (Tsang, et al., 2012). To this end, A431-C7 cells were grown on coverslips and treated with 0.1% Triton buffer or PBS that served as a control, for 6 minutes before fixation. As illustrated in Figure 5.9A, subsequent immunofluorescent staining of treated cells showed a significant reduction in pERM staining and colocalisation with Dsg3 compared to control cells treated with PBS. In support, Western blot analysis of Triton soluble and insoluble fractions from A431-Vect Ct , -D3, -C7 and -C11 cells showed that Dsg3 was distributed in both fractions but predominately distributed in the triton insoluble fraction. Interestingly, pERM proteins were predominantly detectable in the Triton soluble fraction (Figure 5.8B), suggesting complex formation of non-junction Dsg3 with Ezrin. This was substantiated by co-IP that demonstrated clearly that Dsg3 co-IPed with Ezrin in Triton soluble fraction but not in the insoluble fraction (Figure 5.8C). Collectively, these results strongly suggest that non-junctional Dsg3 associates and forms a complex with Ezrin.

5.2.5 Mass spectrometry analysis of the association between Dsg3 and Ezrin.

To identify other candidate proteins that may be in complex with Dsg3, mass spectrometry analysis was carried out on protein complexes isolated using Dsg3 C-terminal truncated protein. To this end, the C-terminus (intracellular domain) of Dsg3 was cloned into a pFN21A *HaloTag* CMV Flexi vector with the *HaloTag* located at the N-terminus (HaloTag-Dsg3 C-terminus design, courtesy of Dr Jaroslaw Szary), a schematic of the construct is illustrated in Figure 5.10A. A431 parental cells were transiently transfected with the C-terminal construct (C-term.Dgs3.*Halo*) and empty vector with *HaloTag* (*Halo.Vet* Ct) which served as a control as described previously. Immunofluorescent microscopy and Western blot analysis of A431-parental cells 48 h after transfection showed positive expression of both *HaloTag.Vect* Ct and the C-term.Dgs3.*Halo* constructs using both the *HaloTag* and Dsg3 C-terminal specific antibody (H145). Specifically, expression of the *HaloTag.Vect* Ct exhibited a band with a

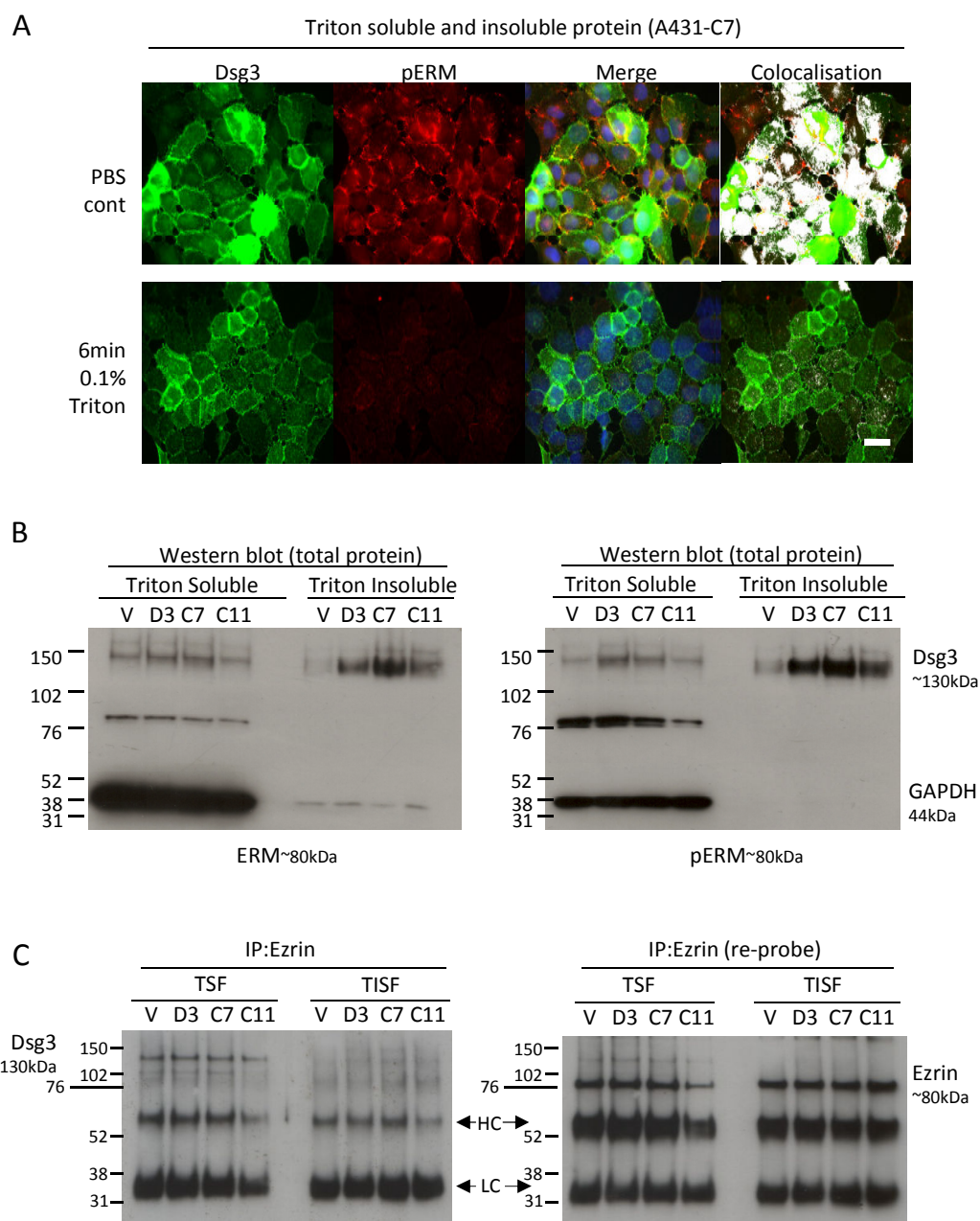


Figure 5.9 Triton soluble pool of Dsg3 colocalises and co-immunoprecipitates with Ezrin. (A) A321-C7 cells seeded on coverslips at 50-60% confluence 24 h prior to treatment with either sterile 1x PBS or 0.1 % Triton X-100 in PBS for 6 minutes. Cells were then washed with PBS before being fixed and immunostained for the indicated proteins. Compared to PBS treated cells, marked reduction was seen in pERM fluorescent staining and to a less degree for Dsg3 in cells treated with Triton. (B) Western blotting and co-immunoprecipitation analyses of Triton soluble (TSF) and insoluble (TISF) fractions of A431 cell lines. Cells seeded at 50-50% confluence 24 h prior to fractionation. The Triton insoluble proteins were dissolved in RIPA buffer. Both fractions were analysed by Western blotting. (C) The fractions of Triton soluble and insoluble protein described in (B) were incubated with the Ezrin antibody and the immune complex was purified using G-protein conjugated beads. Immunoprecipitates were solubilised in protein sample buffer and resolved using SDS-PAGE.

molecular weight of ~37kDa by Western blot while C-term.Dgs3.*Halo* expression showed an approximate molecular weight of 80 kDa (Figure 5.10B). The expression of the C-terminus of Dsg3 did not show any effect on the expression of endogenous Dsg3. Immunofluorescent microscopy was also performed with anti-Dsg3 antibody, 5H10 which recognises an epitope located in the extracellular EC1-EC2 domains. As expected, negative staining using 5H10 was observed in cells expressing the C-terminal construct, nor does it's expression appear to have any effect on the expression of endogenous Dsg3 (Figure 5.10C).

Next, the C-term.Dgs3.*Halo* and *HaloTag.Vect* constructs were transiently transfected in to parental A431 cells and subsequently purified using the HaloTag®Complete pull-down system (Promega) according to the manufacturer's specifications prior to mass spectrometry analyses (courtesy of Dr Min Yang and Miss Isa Cruz, UCL School of Pharmacy). Two independent experiments were performed and relevant proteins were identified by searches using SwissProt human protein databases without restrictions on protein molecular mass or pl. Variable modification of cysteines and oxidation of methionine residues was permitted along with one trypsin mis-cleavage. Peptide and fragment mass tolerances were set at 1+/- Da. Proteins identified with the highest degree of confidence from pull-downs separated by SDS-PAGE were *actin* proteins which were abundantly present in the C-term.Dgs3.*Halo* pull-down and absent and *HaloTag.Vect* Ct pull down (Figure 5.10D). Aside desmosomal components, *Desmoglein 1*, *Desmoplakin* and keratin filament associated protein, *Filaggrin-2*, other proteins identified included *Transcription Factor 7-like 1* which participates in the Wnt signalling pathway, *Heat shock cognate 71kDa* protein (also known as HSC70). The latter has numerous function reviewed by Liu *et al*, in brief, it has been shown to participate in modulating clathrin dynamics (Newmyer and Schmid, 2001) acting as a molecular chaperon in the regulation of cell signalling (Rutherford and Zuker, 1994) and mediating receptor signalling (Fan, et al., 2002). It is also found to be highly expressed in cancer with its knockdown altering tumour cell morphology (Rohde, et al., 2005). A protein identified from pull-downs separated by native gel-PAGE was actin. Ezrin was not detected in the analysis.

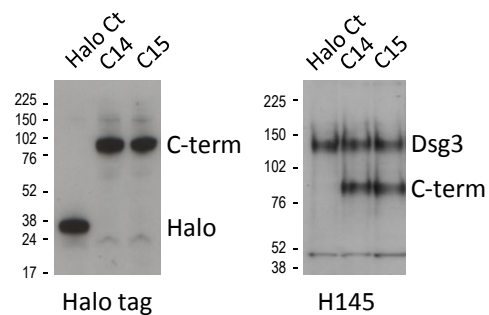
As demonstrated in Figure 5.10C, the expression of C-term.Dgs3.*Halo* exhibited diffused cytoplasmic staining, with no localisation at the plasma membrane. This posed

A

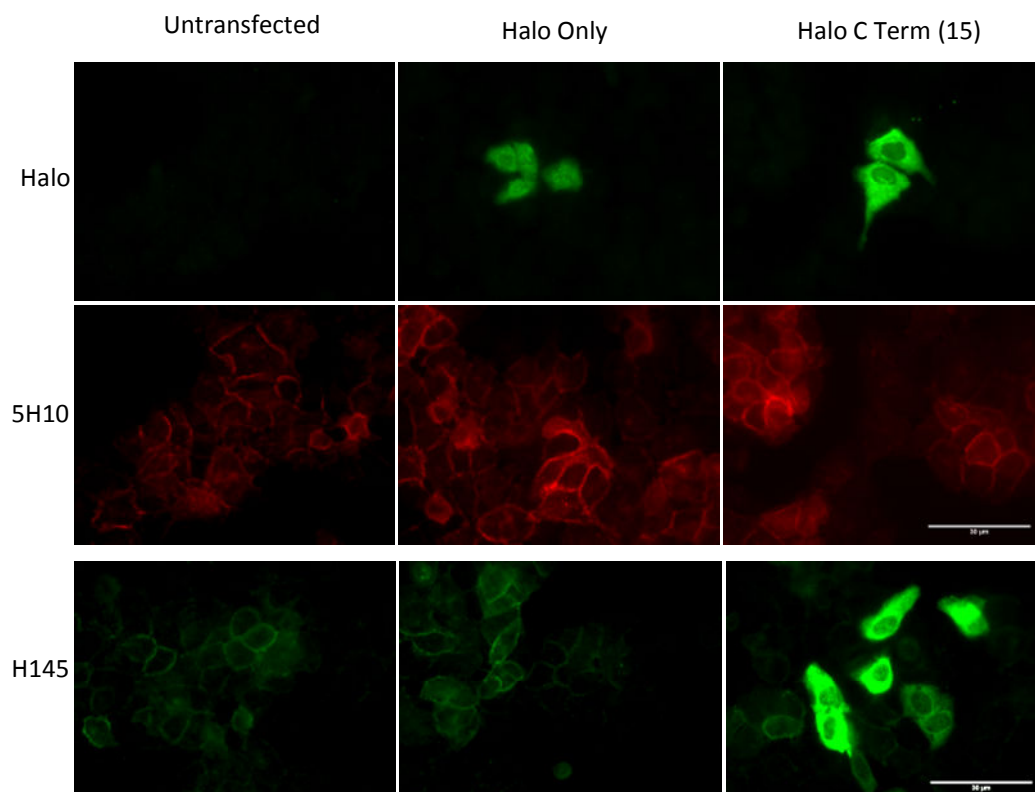
Halo-Dsg3 C-terminal



B



C



D

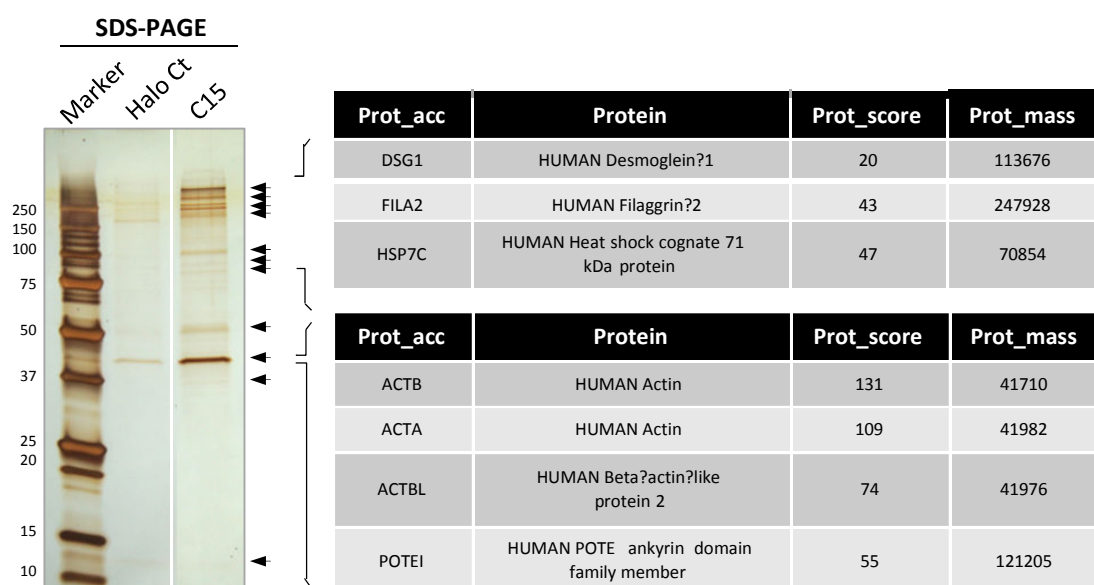


Figure 5.10 Mass spectrometry analysis of isolates from Halo tagged truncated Dsg3 (C-terminus) pull down. (A) Diagram illustrating the construct of Halo:Dsg3 cytoplasmic tail. The antibody binding sites are indicated by double arrows in red. **(B)** Western blotting of lysates of A431 parental cells with transient transfection of the construct shown in (A) (two clones C14 and C15 were tested). Halo vector was used as a control. Truncated Halo:Dsg3 cytoplasmic tail was readily detectable with the Halo tag and Dsg3 antibody H145 that targets to C-terminus of Dsg3. **(C)** Immunostaining of A431-P cells transfected with C-term.Dsg3.Halo using Halo tag, 5H10, and H145 antibodies. scale Bar, 20 μ m. **(D)** Proteomic analysis of protein bands in 1D SDS-PAGE. Actins are found to associate with Dsg3 with the highest scores in the table (Exp n=2). Additional proteins found to be associated with Dsg3 in the native gel include desmoplakin and ubiquinone biosynthesis protein COQ4 homolog (data not shown).

the question whether the localisation of the C-term.Dsg3.Halo protein would affect the profile of proteins it would associate and form complexes with as well as altering its binding capabilities and/or specificities. To address this question mycTag-pull downs from A431-D3 and A431-mycTag Vect Ct cells were analysed as described above. As illustrated in Figure 5.11 Western blot of myc.Tag pull-downs demonstrated sufficient isolation of hDsg3.myc protein from A431-D3 that was further confirmed by immunofluorescence. As illustrated in Figure 5.11B & C, full length Dsg3 associates with the plasma membrane, as described in Chapter 3.

Proteins identified with the highest degree of confidence in isolates separated by SDS-PAGE were *actin* proteins (Figure 5.11D). Other proteins identified included *Plectin*, which acts as a link between the three main components of the cytoskeleton, *i.e.* actin microfilaments, microtubules and intermediate filaments, and has been proposed as a biomarker for pancreatic cancer (Bausch, et al., 2011); *A-kinase anchor protein (AKAP)* *SPHKAP* which acts as a scaffold for the coordination of phospho-events at specific cellular localisation through sequestering kinases and phosphatases with the appropriate substrates; *RhoGEF exchange factor 10-like protein* (GEFs) that stimulates the exchange of GDP for GTP to activate Rho GTPases, Rac1, Cdc24 and RhoA, which in turn bind downstream targets or effector proteins. It is accepted that all Rho GTPases are involved in most, if not all, of the actin-dependent processes, including migration, adhesion and morphogenesis (Chimini and Chavrier, 2000, Kaibuchi, et al., 1999, Luo, 2000). *Unconventional myosin Ic* or class I myosin, that has been shown to be essential to the establishment and maintenance of cortical actin, cell motility and endocytosis was also found. Finally, *Protein Inturned* is implicated in the organization of the apical actin cytoskeleton and the positioning of the basal bodies at the apical cell surface, particularly in ciliated cells, and also an important regulator of Hedgehog (Hh) signal

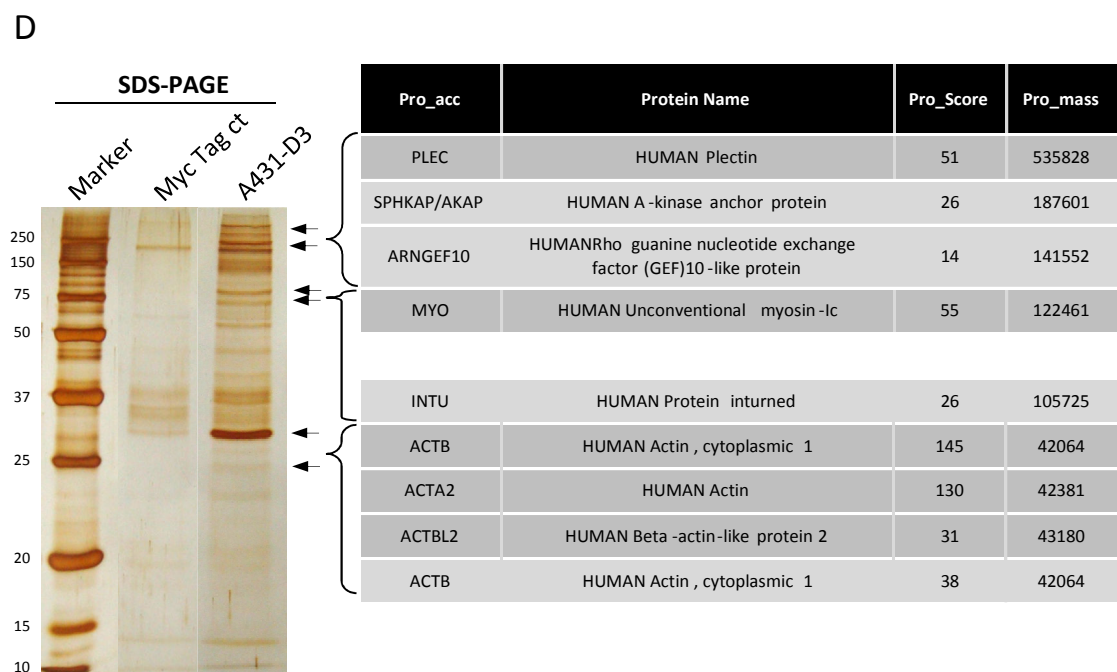
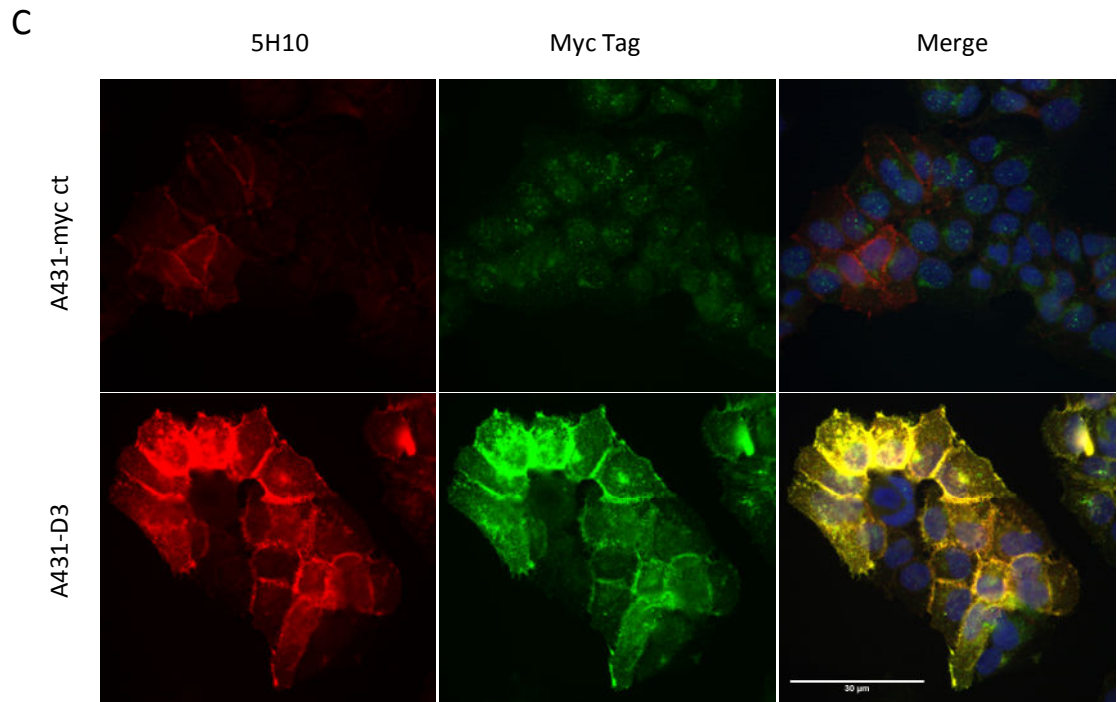
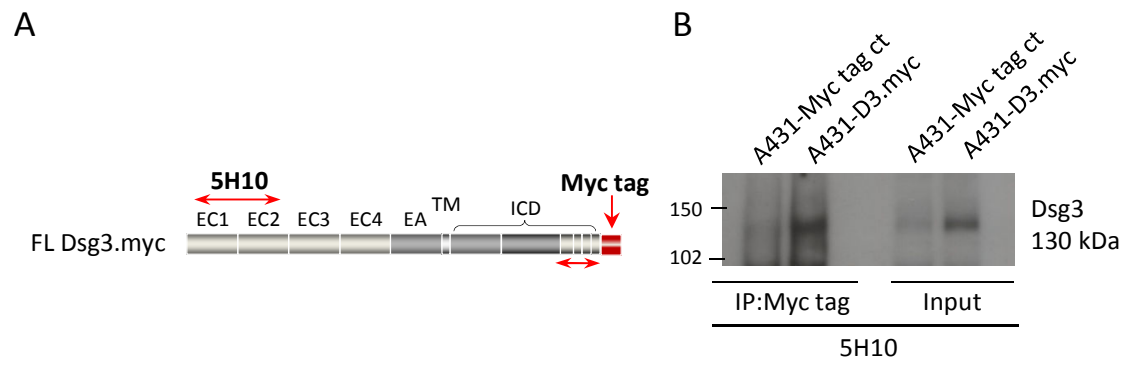


Figure 5.11 Mass spectrometry analysis of isolates from *myc* tagged full length Dsg3 pull downs. (A) Western blot of total cell lysates and those from Myc:tag Immunoprecipitates probed with, Halo tag and H145 antibodies demonstrates the expressions of both the myc:tag Ct and full length constructs **(B)**, supported by Immunostaining using 5H10 and Myc:tag antibodies, scale Bar 20 μ m **(C)**. **(D)** Proteomics analysis of 1D SDS-PAGE gel. Actins are found to associate with Dsg3 with the highest scores (Exp n=1). Additional proteins including RhoGEF exchange factor 10-like protein and GEF 33 and Sodium-dependent phosphate transporter 2 Dsg3 in native gel were identified (data not shown).

transduction and embryonic development (Zeng, et al., 2010). Proteins identified from the native gel –PAGE were actin proteins present in the construct sample and not in the empty vector control, *RhoGEF exchange factor 33*, *Sodium dependent phosphate transporter 2*, *Outer row dynein assembly protein 16* and *DOMO domain-containing protein FRRS1L* (data not shown) were also detected in this analysis. All proteins identified as relevant were deemed so, on the basis that their theoretical molecular weight matched that of the observed molecular weight. However, validation studies are required in order to draw definitive conclusions.

Since actin was identified with the highest degree of confidence in both C-terminus and full length mass spec analysis, to ascertain if they colocalise in *vitro* A431-C7 and -Vect Ct cells where stained for Dsg3, pERM and F-actin and analysed by confocal microscopy. As illustrated in Figure 5.12 all three proteins colocalise predominantly at the plasma membrane, in particular in Dsg3 overexpressing cells (arrows in Figure 5.12A) as well as at membrane protrusion (Figure 5.12B). Interestingly the overexpression of Dsg3 appears to significantly alter the organisation of cortical actin at cell-cell junctions compared to the Vect Ct (Figure 5.12A).

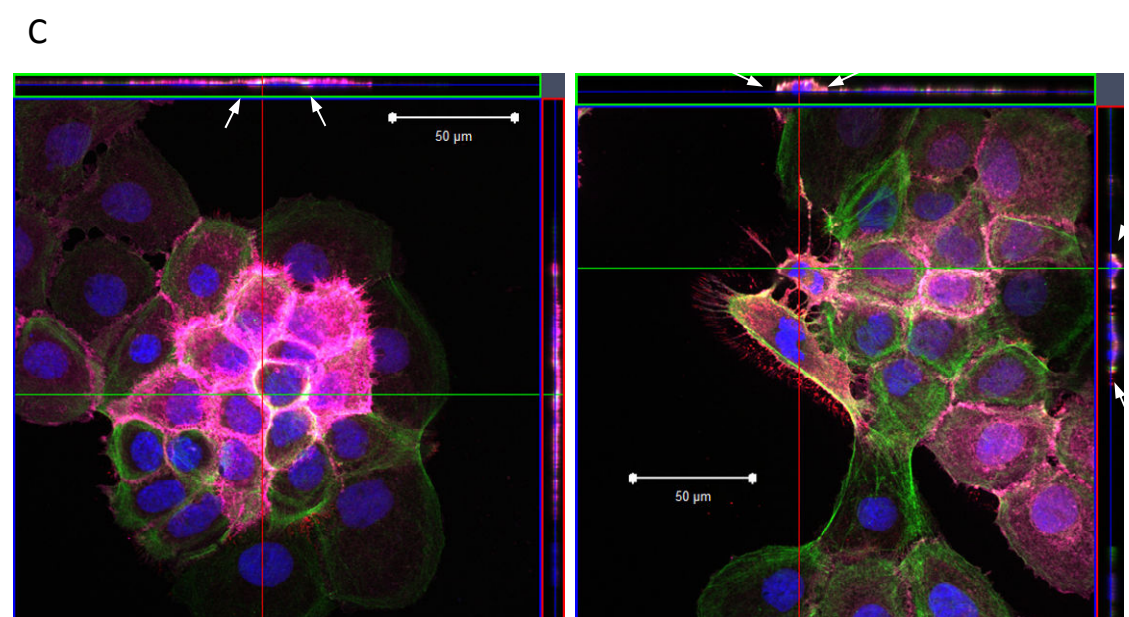
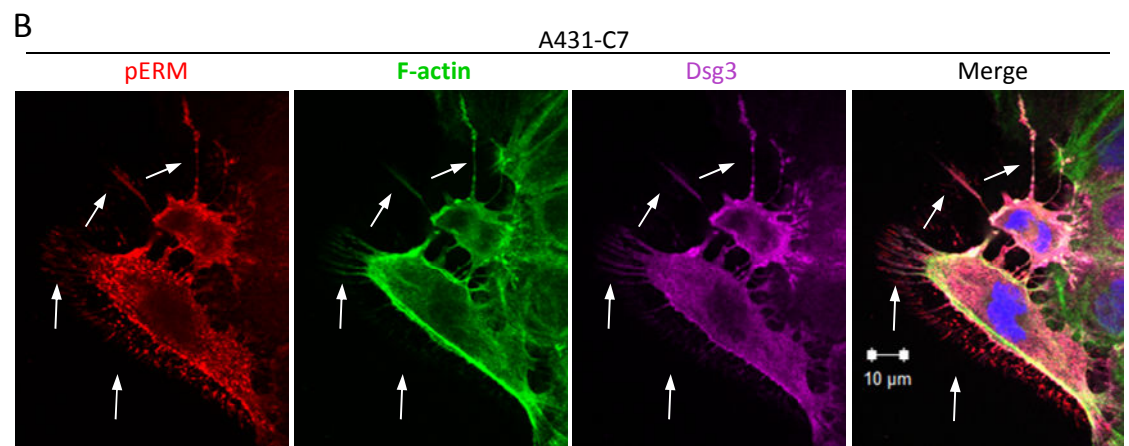
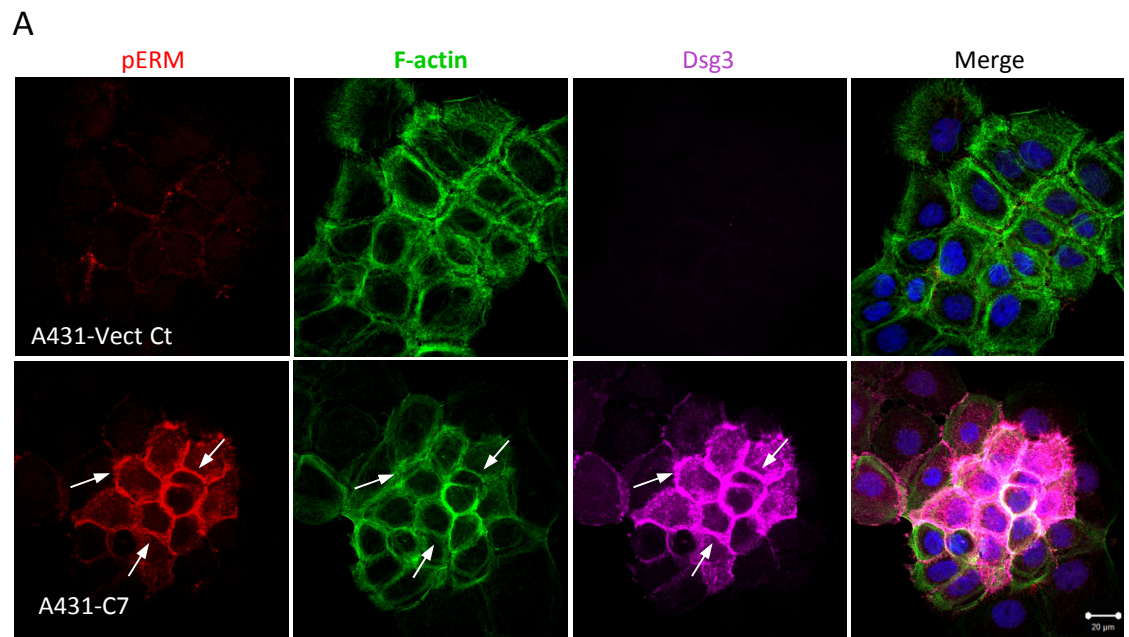


Figure 5.12 Desmoglein 3 colocalise with Ezrin and Actin at cell-cell junctions and membrane protrusions. Confocal microscopy of A431-Vect Ct and -C7 cell lines fixed and stained with Phalloidin, Dsg3 (5H10) and pERM specific antibodies 24 h after seeding at 50-60% confluence on coverslips. Using confocal microscopy Z stacks of 3 fields per coverslip were obtained. **(A)** A comparison of Vect Ct and -C7 cells colocalisation of pERM, F-actin and Dsg3. Colocalisation shown highlighted by white pixels. As illustrated colocalisation occurs at cell-cell junctions and membrane protrusions (white arrows). Scale bare 20µm. **(B)** Colocalisation of pERM, F-actin and Dsg3 at membrane protrusions in A431-C7 focused on membrane protrusions (white arrows). Scale bar 10µm. **(C)** Z stacks of C7 cells shown in (A) & (B) Colocalisation at regions of interest highlight with white arrow., scale bar 50µm

5.3 Discussion

Using a range of technical approaches, this chapter sufficiently demonstrates that non-junctional Dsg3 colocalises and associates with Ezrin, predominantly in membrane protrusions concentrated at the basolateral plasma membrane. Presented here is also evidence that Dsg3 forms a multi-protein complex with proteins that not only facilitate complex formation *i.e.* through anchoring proteins, but also with those essential to the regulation of actin dynamics *i.e.* RhoGEF-like proteins.

The precise function of non-junctional Dsg3 is still unknown. Previous reports provide *in vitro* evidence of an association between non-junctional Dsg3 and intracellular signal proteins including Src (Tsang, et al., 2012). This study confirms that there are two pools of Dsg3 in epithelial cells, consistent with the observations by other research groups (Aoyama and Kitajima, 1999, Baron, et al., 2012, Yamamoto, et al., 2007), and also shows that non-junctional Dsg3 colocalises and possibly forms a complex with Ezrin. Several lines of evidence supports this notion: 1) colocalisation of Dsg3 and Ezrin was demonstrated by fluorescent microscopy, PLA and FRET analyses (Figure 5.1-5.6); 2) complex formation between Dsg3 and Ezrin was reproducibly demonstrated by co-IP in A431-Vect control cells and a variety of cloned cell lines to show a Dsg3-dose-dependent association (Figure 5.7 and 5.9); 3) colocalisation is particularly abundant at membrane protrusions. Taken together these observations suggest that non-junctional Dsg3 possess functions beyond serving a prerequisite pool of desmosome components, through its association with Ezrin in what appears to be specific sub-cellular domains.

An interesting observation was the ability of Dsg3 to form a complex with Ezrin and to a lesser extent Radixin. Interestingly, Ezrin and Radixin both contain a proline rich region, also known as Proline Rich Motif 1 (PRM1) which is absent in Moesin. All proteins that associate with profilin/profilactin (profilin-G-actin complex) have been found to contain the PRM1 motif (Kang, et al., 1997, Purich and Southwick, 1997, Watanabe, et al., 1997). Theoretically, the PRM1 of Ezrin and Radixin would facilitate profilin/profilactin binding which would in turn enable actin filament elongation. However, it is not yet known if such an interaction occurs *in vivo*. If proven, the Dsg3:Ezrin:Profilin/Profilactin complex could play an essential role in the Dsg3-dependent regulation of the actin cytoskeleton.

It is possible that the PRM1 facilitates the association of Dsg3 with Ezrin and Radixin and also explain why Moesin is unable to do so. However, while Ezrin and Radixin appear to have the potential to form complexes with Dsg3. Their cellular localisation will ultimately determine if they will associate and/or form a molecular complex. Staining profiles revealed that while Ezrin and Radixin may both have the potential to associate with Dsg3, Ezrin but not Radixin share a similar cellular distribution to Dsg3 (Figure 5.8). It is important to note that the staining profiles of Radixin and Moesin may be mis-interpreted by the unexpectedly high level of nuclear staining, this is most likely non-specific, due to the known cellular localisation of Ezrin Radixin and Moesin as cytoplasmic (inactive state) and membrane associated (active state) (Hirao, et al., 1996, Takeuchi, et al., 1994). Examination of the images in Figure 5.7 show that all ERM proteins exhibit plasma membrane association with Ezrin the most predominate, followed by Radixin and Moesin which exhibits the least membrane localisation in A431-D3 cells.

In light of a recent study carried out by Fang *et al.*, which observed the increased cytoplasmic colocalisation of Dsg3 in SqCC compared to normal squamous cell epithelia where it adopts a plasma membrane localisation (Fang, et al., 2014). It is possible that through an association with Radixin, Dsg3 could adopt a cytoplasmic localisation, rather than the plasma membrane localisation through Ezrin association (Fang, et al., 2014). In support Intercellular colocalisation between Dsg3 and F-actin has been reported in HaCat cells (Tsang, et al., 2012)

Difficulty in attaining conclusive evidence regarding Ezrin ability to immunoprecipitate with Dsg3, in Dsg3 specific co-immunoprecipitation assay, made for an interesting observation. It is possible that binding of 5H10 antibody to the extracellular region of Dsg3 promotes a conformational change preventing complex formation and Ezrin interaction, this idea is supported by encouraging evidence of complex formation in Myc Tag mediated pull-down. Alternatively it could be attributed to the dynamic nature of the interaction or the interaction occurring between relatively small subset of Ezrin and Dsg3 which may require a more sensitive technique for detection. This may also explain failure to detect Ezrin on Dsg3 pull downs assessed by mass spectrometry.

The cytoplasmic localisation of truncated Dsg3 is most likely attributed to the absence of the transmembrane domain that would ordinarily facilitate its incorporation into the plasma membrane. It would be fair to speculate that the altered cellular localisation would affect the spectra of proteins the truncated C-terminus protein would interact with. Consequently, the profile of proteins identified may not fully reflect the protein interactions *in vivo*. In light of this, mass spectrometry analysis was performed on proteins isolated using full length Dsg3. Interestingly, both actin proteins and RhoGEF-like proteins were identified with the highest degree of certainty in both full length and truncated pull-down. It is likely that these molecules are core components of the Dsg3 complex and facilitate interaction between Dsg3 and Ezrin. In support the colocalisation of actin with Dsg3 have been previously reported at the plasma membrane and cytoplasm (Tsang, et al., 2012) and that Ezrin principally functions through its association with actin (Algrain, et al., 1993, Turunen, et al., 1994). Encouragingly, an interaction between Ezrin and RhoGEF protein, Dbp, has been previously reported in the literature (Lee, et al., 2004, Prag, et al., 2007) to promote directional cell migration in a mechanism involving the downstream activation of Rho GTPases RhoA and Cdc42. These reports support the proposed interaction of Dsg3 with Ezrin and Rho GEF protein(s) as a mechanism to promote cell migration. The presence of AKAP, in the full length Dsg3 immunoprecipitates was a particularly interesting finding due to its orchestrative function, targeting cellular effectors to specific cellular domains including PKA and PKC (Faux, et al., 1999, Klauck, et al., 1996, Rossi, et al., 1999). If the association between Dsg3 and scaffold proteins is proven, they could potentially facilitate Dsg3-dependent signalling and the recruitment of PKC to facilitate Ezrin phosphorylation. Interestingly, it has been reported that Ezrin itself can function as an AKAP selectively binding to the type II regulatory subunit of PKA (Dransfield, et al., 1997). Additionally, AKAPs have also been implicated in orchestrating transduction complexes at the actin cytoskeleton to promote/regulate actin dynamics (Diviani and Scott, 2001, Scott, 2003, Westphal, et al., 2000). While the other proteins identified in complex with Dsg3 support the proposal of Dsg3-dependent signalling and actin regulation capabilities, in order to draw definitive conclusions additional studies would need to be carried out to validate and substantiate such claims.

The identification of actin and RhoGEF-like proteins in both Dsg3 C-terminus and full length pull downs and its absence in control sample is an encouraging result, supporting previous reports that Dsg3 regulates actin organisation and dynamics (Tsang, et al., 2012). Such an event has been suggested by independent researchers focusing on PV pathogenesis demonstrate that PVIgG binding of Dsg3 provokes actin re-organisation (Gliem, et al., 2010). Together, the data presented here and previous chapters suggest that Dsg3 may act as a regulator of the actin cytoskeleton to promote actin based cellular processes including cell migration and invasion, through an association with the actin cytoskeleton and the recruitment of actin regulators to specific sub-cellular domains.

Ezrin was not detected in either the C-term and full length pull downs. This may be attributed to the transient nature of the interaction between Dsg3 and Ezrin and/or the less than adequate optimisation of the steps taken during the isolation Dsg3 complexes. A combination of the both explanations appears plausible with the latter supported by our inability to identify the bait protein, Dsg3. Another consideration is the identification of Keratin associated proteins such as Filaggrin-2 identified in the C-term Pull down and Plectin, Identified in the full length pull down. It cannot be ignored or ruled out that their presence may be attributed to potential contamination of the samples by human skin and hair, which is possible as these pull downs were carried out on the bench. In future pull down assay carried out aseptically.

5.4 Future work

The proximity Ligation assay is a good tool for the quantitation of protein-protein interactions in situ. However, in future the incorporation of a counter stain such as F-actin would allow for the exact cellular localisation of the interactions to be determined. While attempts were made to achieve this, further optimisation was required to determine the best time to incorporate phalloidin Alexa Fluor 488 to attain successful F-actin staining.

The positive control for the PLA does not appear to yield an expected frequency of interactions, given that all exogenous Dsg3 expressed in A431-D3 has a mycTag, I would expect an interaction frequency in excess of the levels detected in the A431-C2 cell for example. I believe that is attributed to the mycTag antibody that was used provided by BD bioscience which lacked sensitivity. This was replaced later during this study by a MycTag antibody provided by cell signalling which exhibited a much higher level of sensitivity. To confirm that this is the reason for the low positive Ct interaction frequency and experiment comparing the two mycTag antibodies will need to be conducted.

To develop the mass spectrometry aspect of the study, further protocols optimisation to ensure the detection of the 'bait' protein, in this case Dsg3, would increase the validity of future findings. Like-wise the development of the FRET using Dsg3 and Ezrin tagged proteins with a FRET pair such as CFP and YFP, or conjugated primary antibodies rather than primary and secondary antibody conjugates would greatly increase the sensitivity of the assay. Additionally the effect of actin disruption on FRET between Dsg3 and Ezrin could be implemented to establish that actin is essential to their association.

Regarding the inability to confirm the ability of Ezrin to co-immunoprecipitate with Dsg3, several things could be adjusted using an antibody which targets other epitopes of Dsg3. Alternatively Co-IP could be carried out on membrane enriched cellular fractions to increase the sensitivity of the assay. Furthermore, the interaction can be analysed using a different approach such as the Co-transfection of Dsg3 and Ezrin cDNA into Cos-1 cells followed by Co-immunoprecipitation analysis.

An interesting aspect of future investigation would be to further substantiate nature of the Dsg3 and Ezrin interaction. For example, to establish if they interact directly or indirectly the yeast 2-hybrid system could be used. If found to be direct, the point of interaction could be further elucidated using a series of blocking peptides. If indirect, the identification of critical binding partners could be confirmed using siRNA the consequences of which could be accessed through the examination colocalisation or FRET between Dsg3 and Ezrin.

Chapter 6: **Desmoglein 3 regulates PKC-dependent Ezrin activity.**

6.1 Introduction

It has been shown that Dsg3 is capable of regulating the actin cytoskeleton (Tsang, et al., 2012), cell morphology and motility (Chapter 4). Chapter 5 provides evidence of a close association and interaction between Dsg3 and Ezrin. Such a close interaction posed the question, whether Dsg3 is in fact functionally regulating Ezrin activity, to modulate the actin cytoskeleton.

The ability of Dsg3 to act as a membrane receptor, mediating signal transduction has been evidenced in pathogenesis studies of PV. Treatment of keratinocytes with Dsg3 auto-antibodies (PVIgGs) have revealed the signalling roles of Dsg3, including its own rapid phosphorylation (Aoyama and Kitajima, 1999), activation of plakoglobin (Pg), PKC, p38 MAPK, heat shock protein p27, Src, Rho A and c-Myc etc. (Berkowitz, et al., 2005, Kawasaki, et al., 2006, Osada, et al., 1997, Waschke, et al., 2006, Williamson, et al., 2006). In addition to the initiation of signalling cascades concurrent cellular events such as an increase in intracellular calcium concentrations (Kitajima, et al., 1999) and actin reorganisation (Gliem, et al., 2010) have been observed. However, the precise role of Dsg3 in regulating these intracellular events and mechanisms involved remains elusive.

Ezrin, as described in Chapter 1, is a membrane cytoskeleton linker protein and plays a role in regulating actin dynamics and the organisation of specialised membrane domains. Ezrin overexpression and phosphorylation at the tyrosine residue 477 and threonine 567 have been shown to be associated with cell migration (Crepaldi, et al., 1997, Donatello, et al., 2012, Lee, et al., 2004, Prag, et al., 2007), cancer progression, metastasis and poor patient prognosis in cancers including breast, pancreas and head and neck squamous cell carcinoma (Chen, et al., 2011, Elliott, et al., 2005, Huang, et al., 2011, Ma and Jiang, 2013, Mak, et al., 2012, Meng, et al., 2010, Saito, et al., 2013, Zhong, et al., 2012). Various kinases, including PKC α (Ng, et al., 2001), PKC θ (Pietromonaco, et al., 1998), Rho-, Cdc42-binding and Nck-interaction kinase (Baumgartner, et al., 2006, Matsui, et al., 1998, Nakamura, et al., 2000) have been shown to directly phosphorylate ERM family proteins. Among them, PKC has been reported to be activated in response to both PVIgG and Ca²⁺ switch (Kitajima, et al., 1999). Furthermore, PKC has also been implicated to participate in actin dynamics (Brandt, et al., 2002), cell motility (Ng, et al., 1999, Xiao and Liu, 2013) and is known to be deregulated in numerous cancer cell lines with its aberrant activation promoting invasiveness while its inhibition perturbed cell invasion (Gopalakrishna and Barsky, 1988, Isakov, et al., 1991, Korczak, et al., 1989, Schwartz, et al., 1990, Takenaga and Takahashi, 1986).

Sharing a common feature, in the regulation of actin-based cellular events, the implication of Ezrin as an effector of Dsg3 through PKC would undoubtedly furnish Dsg3 with the means of regulating cell morphology, migration and invasion. The data presented in pervious chapters characterises the physical relationship between Dsg3 and Ezrin, however, the relationship between Dsg3 and Ezrin activity remains undefined. It is possible that Dsg3, acting as a scaffolding protein, targets Ezrin to specific membrane domains through its recruitment, where Dsg3 elicits Ezrin activation. This chapter focuses on the work undertaken to examine this potential mechanism which proposes that Dsg3 and Ezrin are functionally linked.

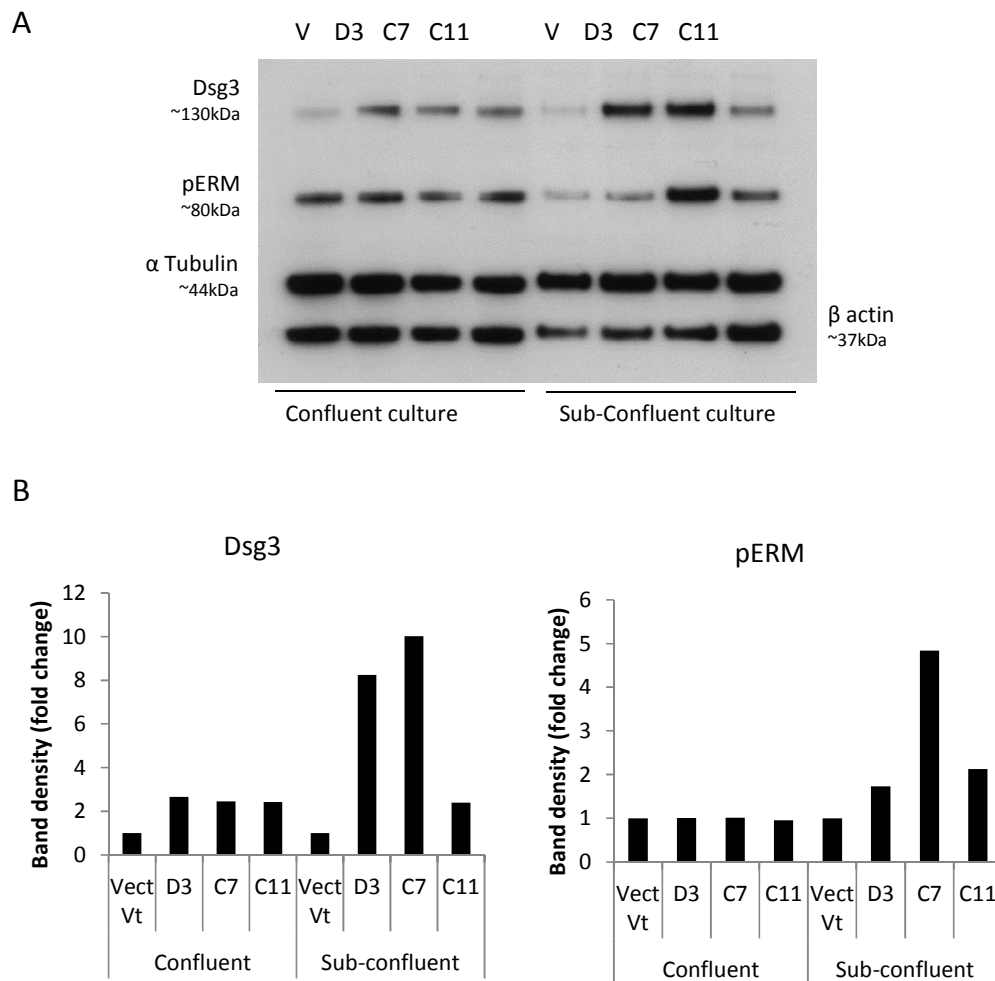


Figure 6.1. Differential Dsg3 expression and ERM protein phosphorylation in sub-confluent and confluent culture. Western blot of total cell lysates of A431-Vect Ct (V), D3, C7 and C11 that were seeded to achieve confluent and sub-confluent density **(A)**. **(B)** Quantitation of 2 experiments comparing confluence and sub-confluent culture conditions. Both western blots were normalised against α tubulin and relative Vect control level set at 1 arbitrary unit.

6.2 Results

6.2.1 Overexpression of Dsg3 increases the phosphorylation of Ezrin at Threonine 567.

It has been shown that the phosphorylation of Ezrin at conserved C-terminal threonine residue 567 (Thr567) confers activation and is implicated in tumour cell migration, invasion and metastasis (Ng, et al., 2001). In the search for biomarkers in cancers, other independent studies have discovered that Dsg3 is up-regulated in squamous cell carcinoma in various organs of the body, particularly in lung and the lymph node with metastases in head and neck cancer (Patel, et al., 2013). To characterise the relationship between Dsg3 and Ezrin activity the level of Ezrin phosphorylation at

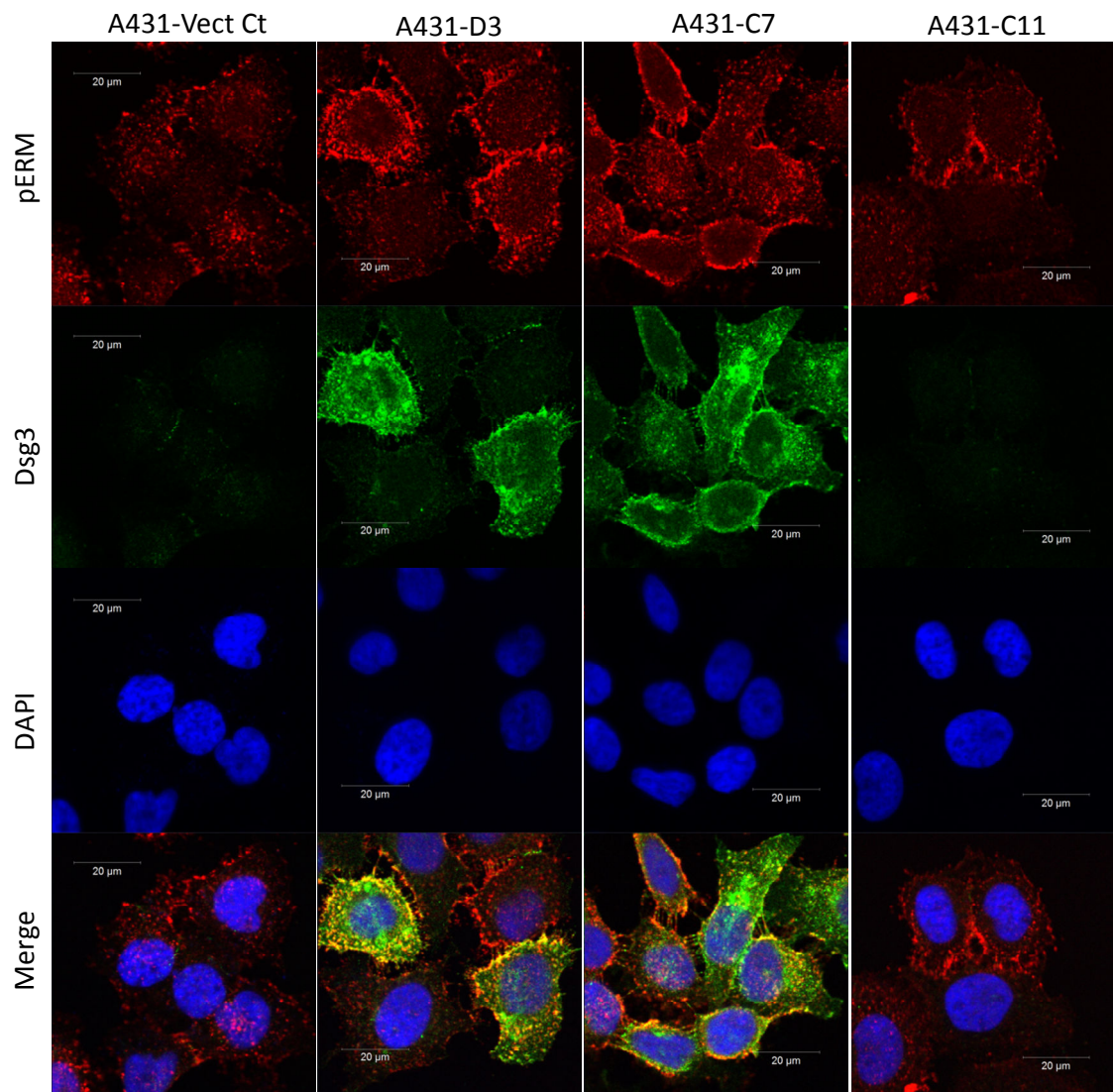


Figure 6.2 Overexpression of Dsg3 enhances ERM protein phosphorylation assessed by fluorescence microscopy analysis. A431 cell lines were seeded on coverslips at 50-60% confluence 24 h before fixation and immunostained for the indicated proteins. Colocalisation in merged channels is highlighted by the yellow/orange pixels. Four fields for each cell line were acquired. Illustrated are representative images for each cell line. $n = 5$, Scale bar 20 μm .

Thr567 with respect to Dsg3 expression was examined. For this purpose, a rabbit polyclonal antibody that detects phosphorylated Ezrin, Radixin and Moesin at Threonine 567, 564 or 558, respectively [Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558), denoted as 'pERM'], was employed.

In order to establish the cellular conditions that would promote Dsg3-dependent phosphorylation of ERM proteins, Dsg3 expression and ERM protein phosphorylation were examined in both confluent and sub-confluent cultures. Here, total cell lysates from A431-Vect Ct, -D3, -C7 and -C11 cells, seeded to achieve confluent and sub-

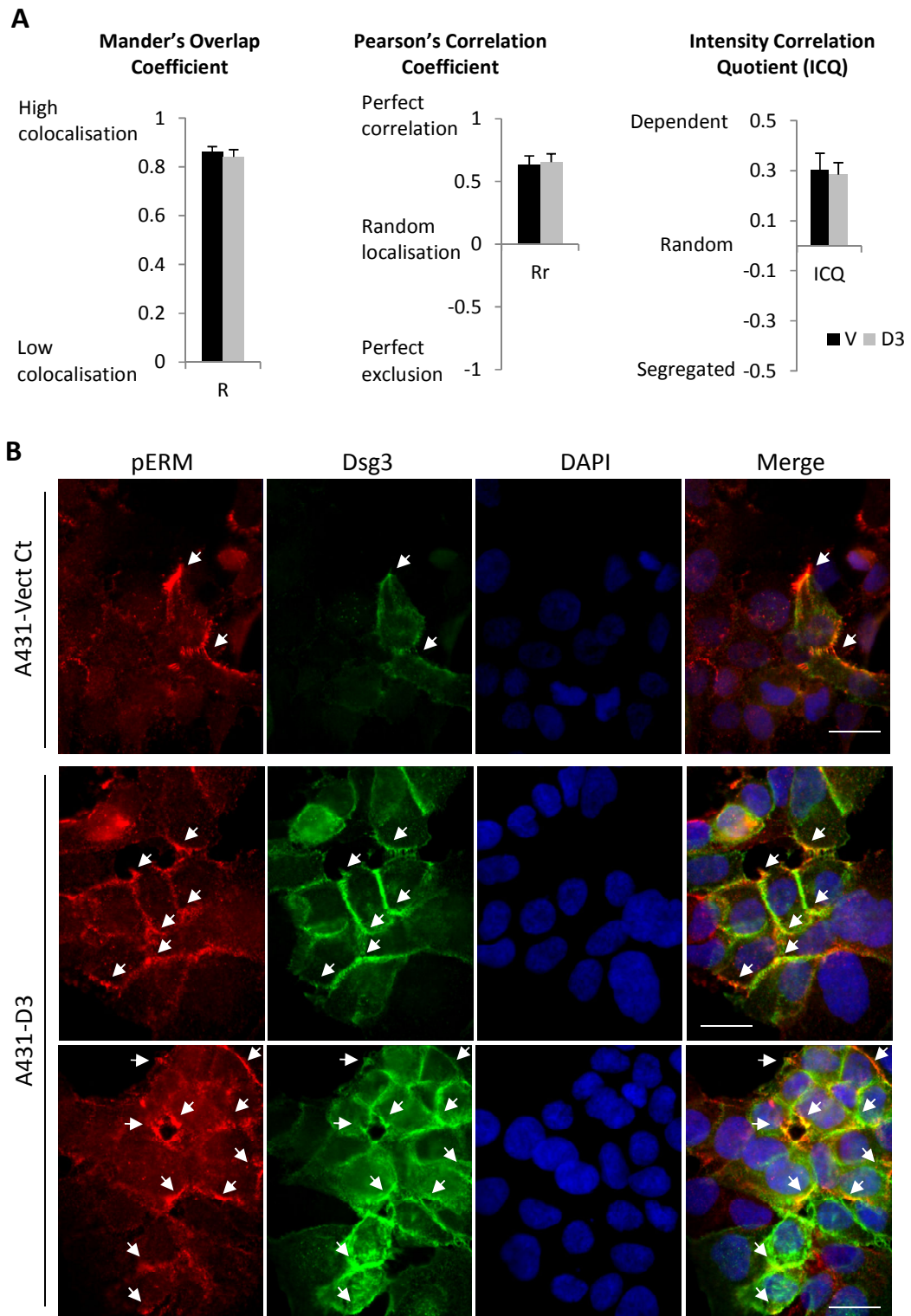


Figure 6.3 Colocalisation between Dsg3 and pERM are equally significant in A431-Vect Ct and A431-D3 cells. A431 cell lines were prepared and processed as previously described. Using the ImageJ colocalisation analysis plug-in, Pearson's correlation Coefficients (Rr), Mander's Overlap Coefficients (R) and Intensity Correlation Quotient (ICQ) were determined for Dsg3 and pERM staining. Two independent experiments were performed and data were representative (coefficient mean value \pm SD) **(A)**. **(B)** Representative images of A431-Vect Ct and -D3 cells show colocalisation between both exogenous and endogenous Dsg3 with pERM (white arrows). Scale bar 20 μ m

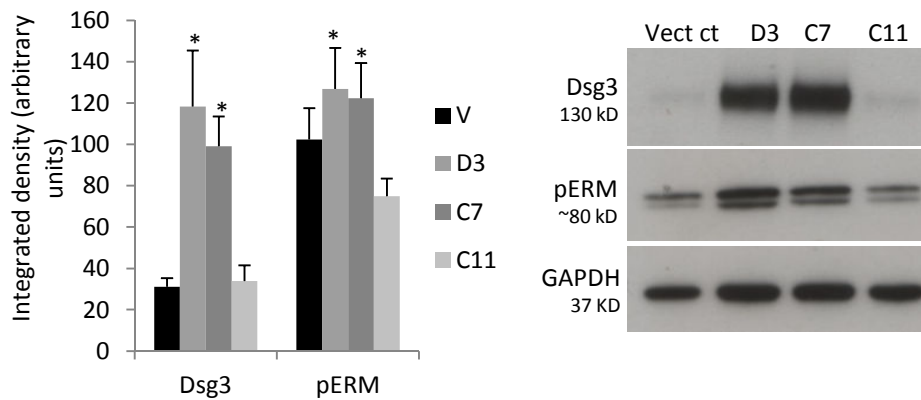


Figure 6.4. Overexpression of Desmoglein 3 enhances the ERM protein phosphorylation assess by Western blot. Western blot of total cell lysates of A431 cell lines seeded at 50-60% density for 24 h showed increased Dsg3 expression correlated with elevated levels of pERM. Band density of blots were determined using ImageJ gel analysis tool normalised against GAPDH. (n=7, * $p < 0.05$).

confluent culture 24 h prior, were extracted followed by Western blot analysis. As illustrated in Figure 6.1, culture confluence had an apparent effect on both Dsg3 expression and ERM protein phosphorylation. In confluent culture Dsg3 expression and ERM phosphorylation were suppressed and exhibited little sample variation. In sub-confluent culture however, the cells exhibited differential Dsg3 expression and Dsg3-dependent ERM protein phosphorylation. In support, fluorescent microscopy was performed on A431 cell lines labelled with Dsg3 and pERM specific antibodies. As shown in Figure 6.2, a significant increase in pERM staining was observed in cell lines with high Dsg3 expression, A431-D3 and -C7 ($p < 0.01$), compared to the Vect Ct, suggesting a dose-dependent correlation between Dsg3 expression and the phosphorylation of ERM proteins ($p < 0.01$, Figure 6.2). An assessment of percentage colocalisation using ImageJ in these cell lines demonstrated a significant increase in the colocalisation between Dsg3 and pERM (>5-fold) in A431-D3 and -C7 cells compared to -Vect Ct ($p < 0.001$, Figure 6.2). To confirm the colocalisation exhibited in Vect Ct and D3 cell were equally significant, *Pearson*, *Manders* and *ICQ coefficients* as described in Chapter 5, were performed using ImageJ and showed that Dsg3:pERM staining was dependent, synchronous and colocalised in both Dsg3 low (A431-Vect Ct) and Dsg3 high (A431-D3) expressing cells (Figure 6.3). In further support, Western blot analysis showed consistently a significant increase in ERM protein phosphorylation in Dsg3 overexpressing cells compared to Vect Ct and C11 cells with a clear dose-dependent

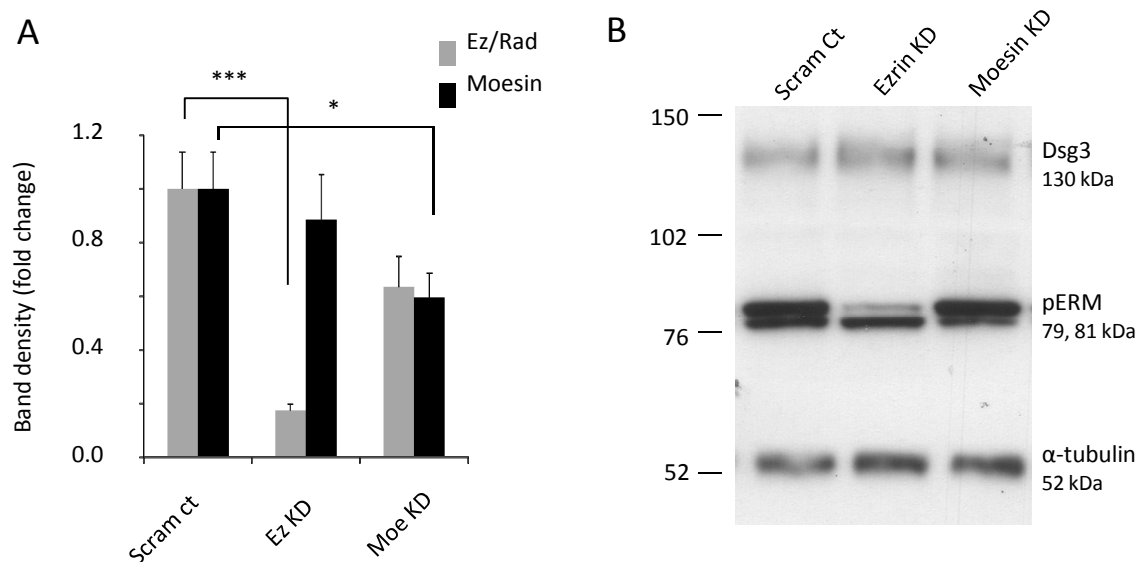


Figure 6.5 Ezrin is the predominate ERM protein hyper-phosphorylated in response to Dsg3 overexpression. A431-C7 cell lines were seeded 24 h prior to treatment with either Ezrin, Moesin specific or Scrambled Ct siRNA. A further 24 h after siRNA treatments. **(A)** Quantitation from 3 independent experiments. Blot densitometry: normalised against α Tubulin and relative Scrambled control level set at 1 arbitrary unit **(B)** Illustrates a representative Western blot of total cell lysates was carried out with the indicated antibodies (Exp n=3, * $P < 0.05$, *** $P < 0.001$).

correlation with respect to Dsg3 (a representative of seven independent experiments is shown in Figure 6.4).

6.2.2 Ezrin is the predominant ERM protein in A431 cells.

The use of pERM antibody as a means of assessing Ezrin phosphorylation is limited due to the inability of distinguish between phospho-Ezrin (Thr567), phospho-Radixin (Thr564) and phospho-Moesin (Thr558). To determine which of the ERM protein predominantly attributes to Dsg3-dependent increases ERM phosphorylation, a knockdown experiment for the individual ERM proteins was carried out. Here, silencing was restricted to Ezrin and Moesin, the predominant ERM proteins in A431 cells (Yonemura and Tsukita, 1999). In this analysis, A431-D3 cells, prepared a day prior, were transiently transfected with Ezrin Moesin specific and scrambled Ct siRNAs. Cells were re-seeded 24 hrs after transfection followed by lysate extraction and Western blot analysis. Figure 6.5 shows that cells transfected with scrambled siRNA exhibited typical double bands of pERM in A431 cells, with the upper band being attributed to Ezrin and Radixin (~80 kDa) and the lower band attributed to Moesin (~75 kDa). Importantly, knockdown of Ezrin resulted in the most significant reduction of upper

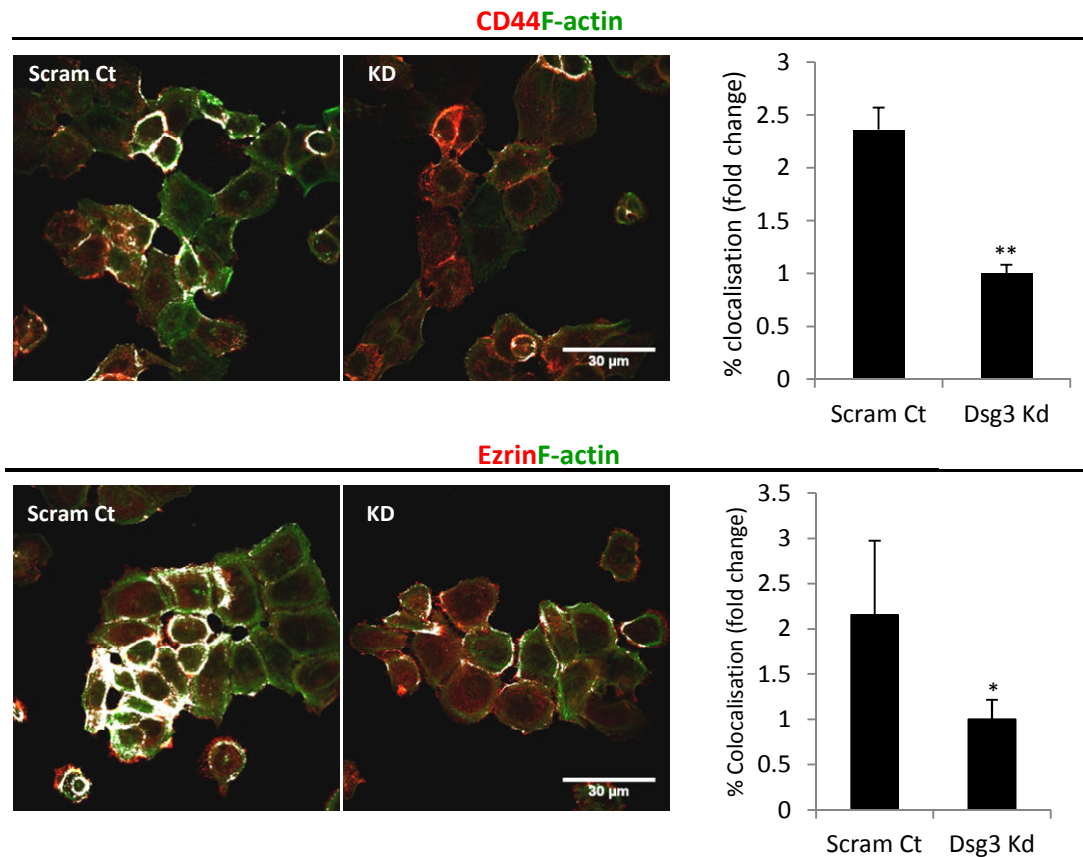


Figure 6.6 Desmoglein 3 silencing reduces CD44:F-actin and Ezrin:F-actin colocalisation. A431-D3 cell lines were seeded 24 h prior to treatment with either Dsg3 specific or Scram Ct siRNA. Cells were re-seeded 24 h after transfection on coverslips followed by fixation and immunostaining for the indicated proteins 24 h later. At least 3 fields per coverslip were acquired with a confocal microscope and analysed using ImageJ to determine the percentage colocalisation between the labelled proteins as shown in bar charts on the right. (n=2, * $P < 0.05$, ** $P < 0.01$).

band density (~75%) compared to control and also that of the Moesin knockdown (~10% reduction), implying that Dsg3-dependent increases in ERM protein phosphorylation is predominately attributed to Ezrin phosphorylation. Additionally,

6.2.3 Knockdown of Dsg3 reduces Ezrin activity.

To address the question whether Dsg3 knockdown attenuates ERM phosphorylation, Western blot and immunofluorescence analyses were performed on A431-D3 cell transiently transfected with Dsg3-specific and Scrambled siRNA as described above. No detectable reduction in ERM protein phosphorylation was evident following Dsg3 silencing (Figure 6.7).

As discussed in Chapter 1, upon activation, Ezrin functions as a regulated linker between the plasma membrane to the actin cytoskeleton through direct interaction

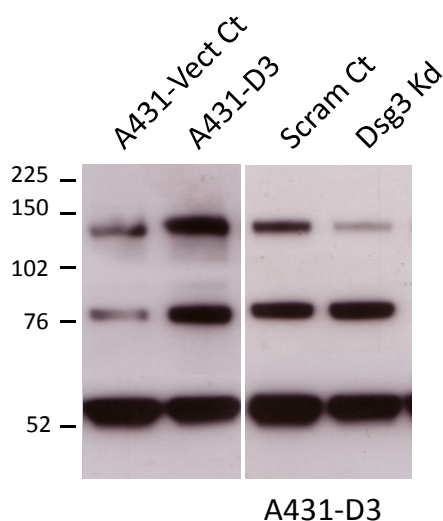


Figure 6.7. Desmoglein 3 knockdown appears to have no significant affect on ERM protein phosphorylation. Western blot analysis of Dsg3 knockdown and scrambled Ct A431-D3 cells prepared as previously described reveals. Illustrated are representative images of A431-Vect Ct and A431-D3 cells and A431-D3 cells subjected to either Scrambled Ct or Dsg3 specific siRNA ($n \geq 3$)

scrambled siRNA 48h prior to immunostaining stained for CD44:F-actin and Ezrin:F-actin. Colocalisation analysis of these images using ImageJ renders overlapping pixels in white. As shown the knockdown of Dsg3 significantly reduces the level of colocalisation between of F-actin:Ezrin and F-actin:CD44 by ~2-fold compared to the scrambled control, suggesting that Dsg3 function regulates Ezrin activity at the plasma membrane.

6.2.4 Desmoglein 3-dependent ERM phosphorylation could be abrogated by inhibiting a variety of signal molecules.

In order to elucidate the possible signalling mechanism(s) involved in Dsg3-mediated Ezrin activation/regulation, a pharmacological based study was performed to examine the effect of specific molecular inhibition on Dsg3-dependent ERM protein phosphorylation. In preparation, A431-C7 cells were seeded and allowed to adhere before being serum starved overnight. The following day cells were treated with a variety of pharmacological inhibitors that target various signal molecules including isoform-specific PKC inhibitors Bisindolymaleimide I HCl, Gö 6976 and RÖ 31-7549. Bisindolymaleimide I HCl (BIM) is a potent broad spectrum competitive inhibitor for the ATP-binding site of PKC, highly selective for classical isoforms

(Martiny-Baron, et al., 1993), Gö 6976 selectively inhibits classical isoforms,

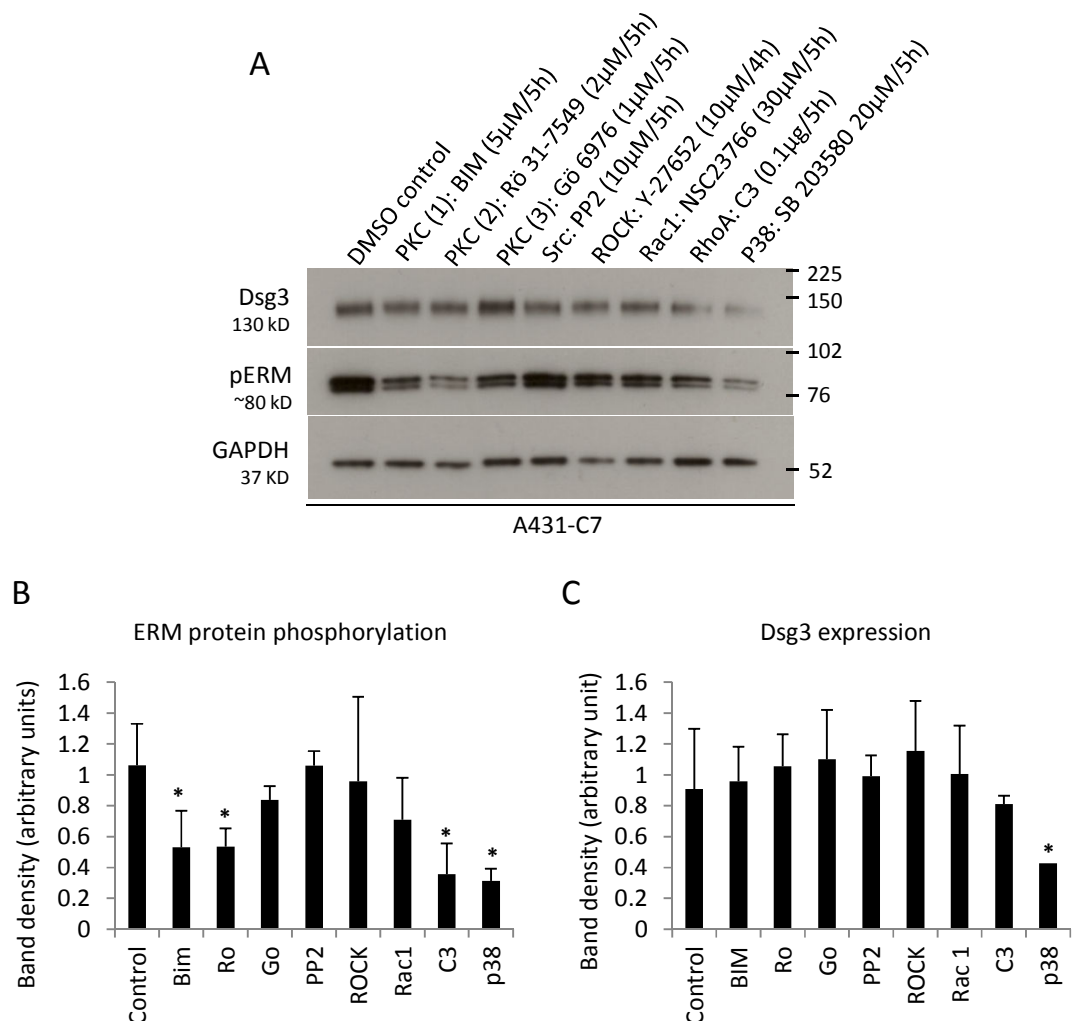


Figure 6.8 Desmoglein 3–dependent ERM protein phosphorylation is inhibited by PKC, RhoA and p38 inhibition. A431-C7 cells were seeded at 50–60% confluence and allowed to adhere for 4–6 h before serum starved overnight. Cells were then treated with various inhibitors or vehicle control in media supplemented with 10% serum for ~4 h. **(A)** Western blot of total cell lysates. Quantitation of relative Dsg3 expression **(B)** and ERM protein phosphorylation **(C)** were shown in the bar charts showing relative band density, normalised against GAPDH (n=4, *P<0.05).

particularly PKC α and PKC β 1 with no effect on calcium independent isoforms (Martiny-Baron, et al., 1993) and Rō 31-7549 selectively inhibits all classical isoforms as well as novel PKC ϵ which requires DAG but not Ca²⁺ for activation (Wilkinson, et al., 1993). Other inhibitors employed include those targeting Src, ROCK, Rac1 and MAPK p38, all of which have been implicated in Dsg3 signalling and ERM protein activation (Auvinen, et al., 2007, Hébert, et al., 2008, Kawasaki, et al., 2006, Menager, et al., 1999, Tsang, et al., 2012, Tsang, et al., 2010). The inhibitor solvent, DMSO, was used as a vehicle control in these analyses. Cells were incubated with an inhibitor diluted in normal growth media for 3–4 h (see Table 2.2 for inhibitor-specific incubation periods)

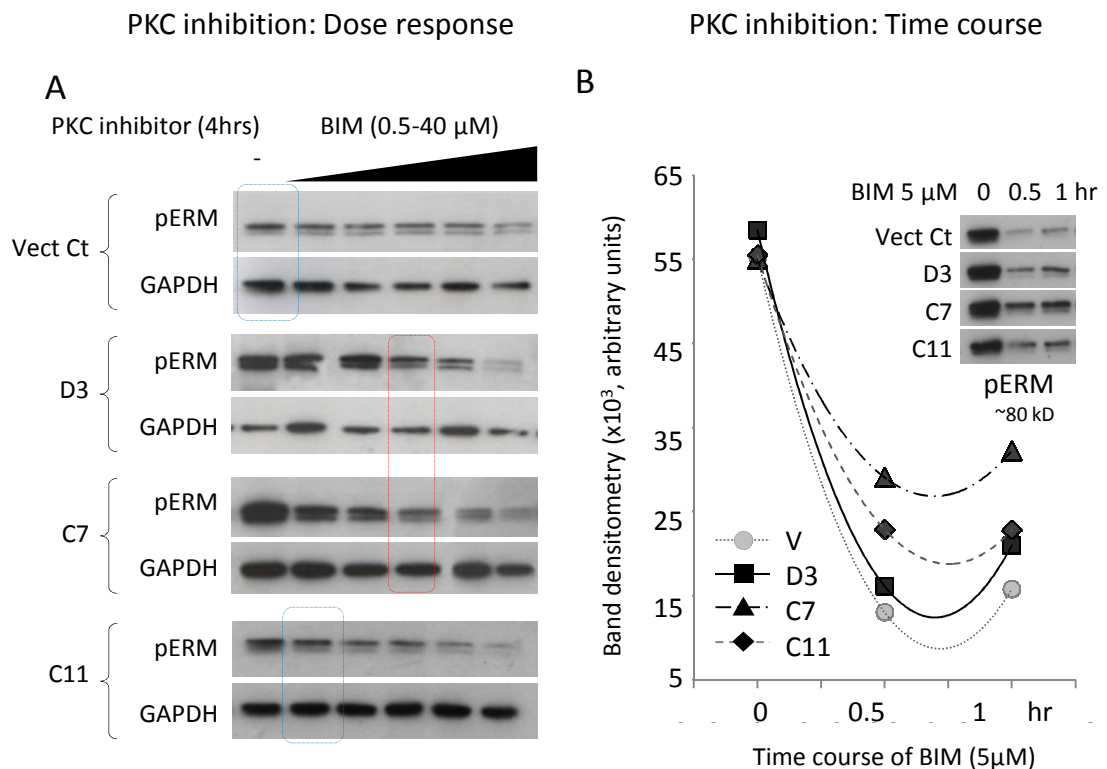


Figure 6.9 Abrogation of ERM protein phosphorylation by PKC inhibition occurs in a Dsg3-dose dependent manner. Western blotting of total cell lysates of the cell lines indicated seeded at sub-confluence and allowed to adhere for 4-6 h before serum starvation overnight. Cells were then treated with inhibitors or vehicle control (DMSO) diluted in media supplemented with 10% serum at concentration and time points indicated. **(A)** A higher concentration of BIM was required to bring ERM phosphorylation levels of A431-D3 and -C7, (Red box) Similar to those of the Vect Ct and low expression cell population -C11 (Blue boxes). **(B)** When exposed to fixed concentration of BIM, cells overexpressing Dsg3 (A431-D3 and -C7) maintain a higher level of ERM protein phosphorylation than those of the Vect Ct and low expression cells, C11. **(A)** n=3, **(B)** n=3).

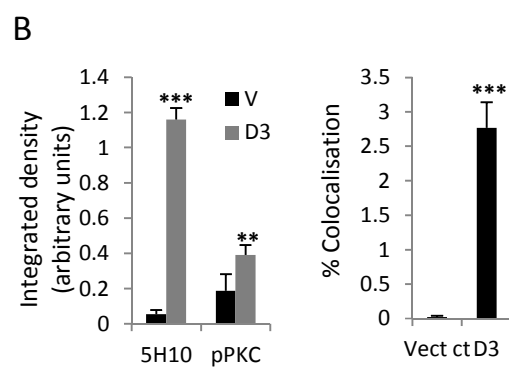
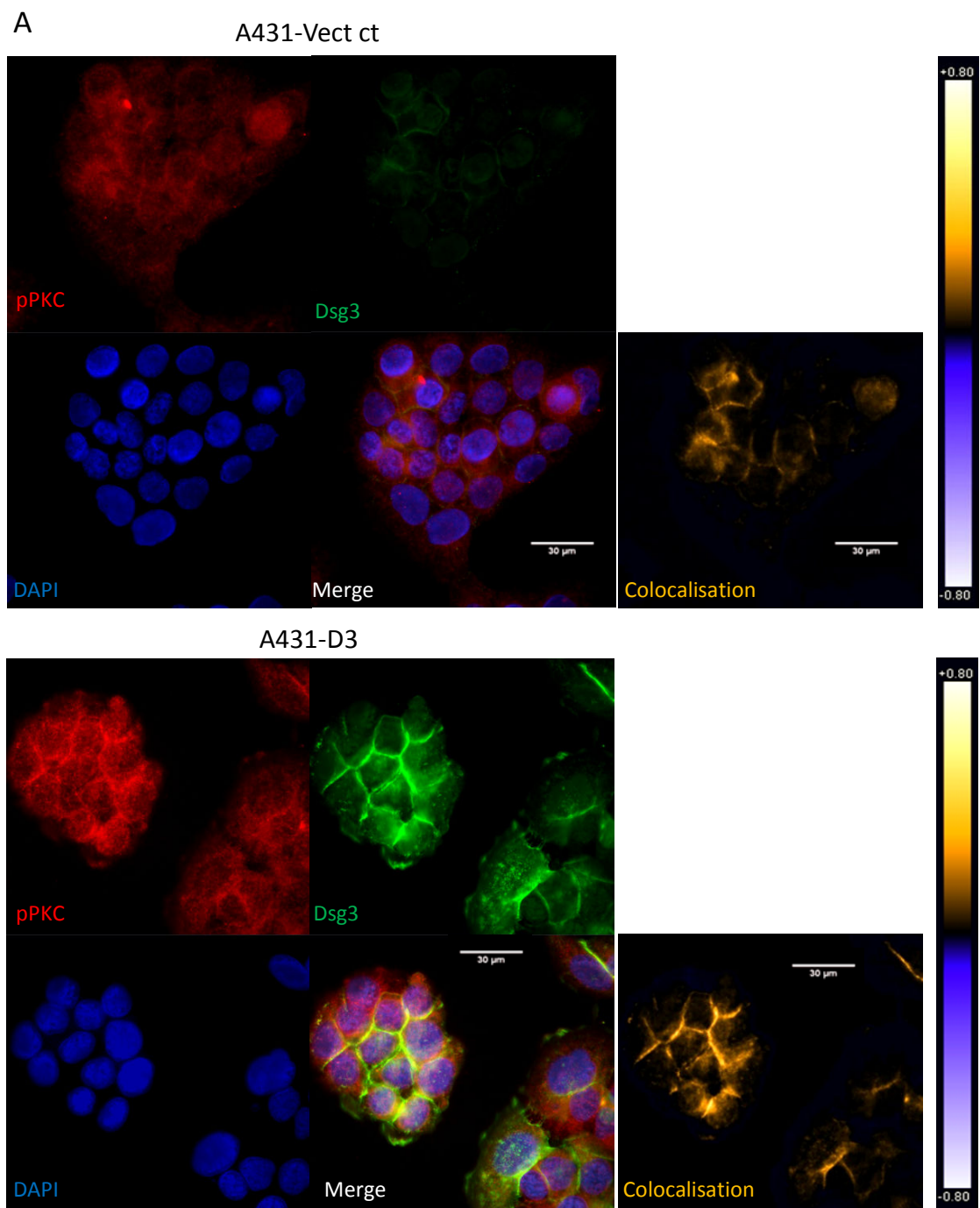
followed by cell lysate extraction. Lysates were then analysed by Western blot for Dsg3 and pERM. As illustrated in Figure 6.8 the inhibition of PKC, RhoA, ROCK, Rac1 and p38 resulted in a significant reduction in Dsg3-dependent ERM phosphorylation. Interestingly, the inhibition of p38 MAPK also resulted in a significant reduction in Dsg3 expression, implying a negative feedback loop suppressing Dsg3 expression downstream of p38. Together, these results suggest that PKC, Rac1, RhoA and p38 signalling pathways play a role, in concert, in facilitating Dsg3-dependent Ezrin phosphorylation in A431 cells

6.2.5 Desmoglein 3-dependent ERM phosphorylation is abrogated by PKC inhibition.

PKCs are the characterised serine/threonine kinases that directly regulate ERM phosphorylation (Ng, et al., 2001, Pietromonaco, et al., 1998, Wald, et al., 2008) and identified to act as downstream effectors of Dsg3 (Kawasaki, et al., 2006, Kitajima, et

al., 1999). To further investigate whether Dsg3-mediated Ezrin activation was PKC-dependent, dose-response and time-course assays were performed using A431 cells treated with the broad spectrum PKC inhibitor, BIM. In preparation for the dose-response assay, A431-Vect Ct, -D3, -C7 and -C11 were seeded for ~4 h and serum starved overnight. The following day cells were treated with BIM at a series of increasing concentrations (0.5, 5, 10, 20, 40 μ M) or DMSO vehicle control diluted in normal growth media for 4 h. Following treatment cell lysates were extracted and analysed by Western blot for Dsg3 and pERM. As illustrated clearly in Figure 6.9A, a higher concentration of BIM (red box) was required to bring the levels of ERM protein phosphorylation down to that of Vect control or C11 cells treated with less concentration of the drug (blue boxes). This suggests that Dsg3-dependent ERM phosphorylation involves PKC and that PKC activity is modulated by Dsg3. Similar results were shown in a time-course study where A431-Vect Ct, -D3, C7 and -C11 cells, prepared in the same way, were treated with a fixed concentration of BIM (5 μ m) with cell lysates extracted after 0.5 and 1 h incubations. Lysates extracted at time point zero served as a control. As illustrated in Figure 6.9B, an observable slight increase in ERM protein phosphorylation at 0 h time point was evident in A431-D3 and -C7 cells compared to Vect Ct and C11 cells. A significant reduction of pERM was seen in all cell lines treated with inhibitor for both 0.5 h and 1 h incubation periods. On closer inspection, the degree of inhibition of ERM phosphorylation was not occurring at the same degree amidst different cell lines, with the level of ERM phosphorylation inhibition observed in A431-D3 and -C7 cells comparatively less than that of the Vect Ct and C11 cells (Figure 6.8B). This results show that cells with high Dsg3 expression retained higher levels of pERM than those with low Dsg3 expression, suggesting a positive correlation between Dsg3 expression and PKC activity, with Dsg3 sequestering PKC to phosphorylate (activate) ERM proteins in a dose dependent fashion. Together, these results suggest that Dsg3-mediated Ezrin phosphorylation is mediated through PKC.

It has been shown that PKC isoforms localise in different sub-cellular compartments indicative of isoform specific function (Kiley and Parker, 1995). To examine the cellular localisation of PKC with respect to Dsg3, immunostaining of A431-Vect Ct and -D3 cells was performed using Dsg3 and pan-phospho-PKC (pPKC) antibodies. The results



C

A431	Rr	R	ICQ
Vect ct (sd)	0.850 (± 0.015)	0.929 (± 0.006)	0.406 (± 0.009)
D3 (sd)	0.756 (± 0.032)	0.797 (± 0.020)	0.384 (± 0.012)

Figure 6.10 Dsg3 colocalises with PKC at the plasma membrane in a dose dependent mannerr. A431-Vect Ct and D3 lines seeded at 50-60% density were fixed and immunostained for the indicated proteins 24 h after seeding. Here pPKC denotes pan-phospho-PKC. Image analysis was carried out using ImgaeJ to determine relative integrated density **(B)**, percentage colocalisation **(B)** and various colocalisation coefficients (R_r =*Pearson's correlation coefficient*, R =*Mander's Overlap coefficient*, ICQ =Intensity Correlation Quotient in the table) **(C)**. **(A)** Representative images of A431-Vect Ct and –D3 cells colocalisation images show only colocalisation pixels (Orange) (n=2, $**P<0.01$, $***P<0.001$). Scale bar, 30 μ m.

predominately at the plasma membrane, with a significant increase in percentage colocalisation in A431-D3 compared to Vect Ct (Figure 6.10). Further analysis of the colocalisation in both cell lines using *Manders*, *Peasons* and *ICQ* coefficients showed that the colocalisation in both Vect Ct and –D3 cells is of equal significance (Figure 6.10 Table). Currently there are 15 characterised PKC isoforms which can be classified into 3 groups, *i.e.* conventional, novel and atypical. Conventional isoforms include α (alpha), β (beta), γ (gamma) that require DAG (Diacylglycerol), Ca^{2+} and phospholipids for their activation. Novel isoforms comprise δ (delta), ϵ (eplison), η (eta), and θ (theta) that require DAG but not Ca^{2+} for their activation and Atypical isoforms including ι (iota), ζ (zeta) and PKN (serine/threonine protein kinase) that require neither DAG nor Ca^{2+} for their activation. In order to determine which group of PKCs was engaged in Dsg3 mediated Ezrin phosphorylation (activation), an experiment with various PKC inhibitors was performed in A431-C7 cells either in the presence or absence of calcium. The calcium chelating agent, EGTA, has been reported to have the similar effect to that of pharmacological inhibition of PKC (Hui, et al., 2006). Assuming that classical isoforms of PKC were involved, depletion of extracellular calcium should reduce ERM protein phosphorylation. To test this hypothesis three PKC inhibitors were employed, the broad spectrum PKC inhibitor BIM (Toullec, et al., 1991), Gö 6976 for classical isoforms (Martiny-Baron, et al., 1993) and Rö 31-7549 a broad spectrum PKC inhibitor with a preference to classical isoforms, particularly PKC alpha (Wilkinson, et al., 1993). As illustrated in Figure 6.10, the addition of calcium caused a significant increase in Dsg3 expression, as expected. In relation to ERM phosphorylation, calcium switching appeared to have little or no apparent effect on the level of phosphorylation. The inhibition of PKC did not appear to have any effect on Dsg3 expression in the presence or absence of calcium (Figure 6.11). However, only in the absence of calcium did all three inhibitors bring about a significant reduction in ERM protein phosphorylation

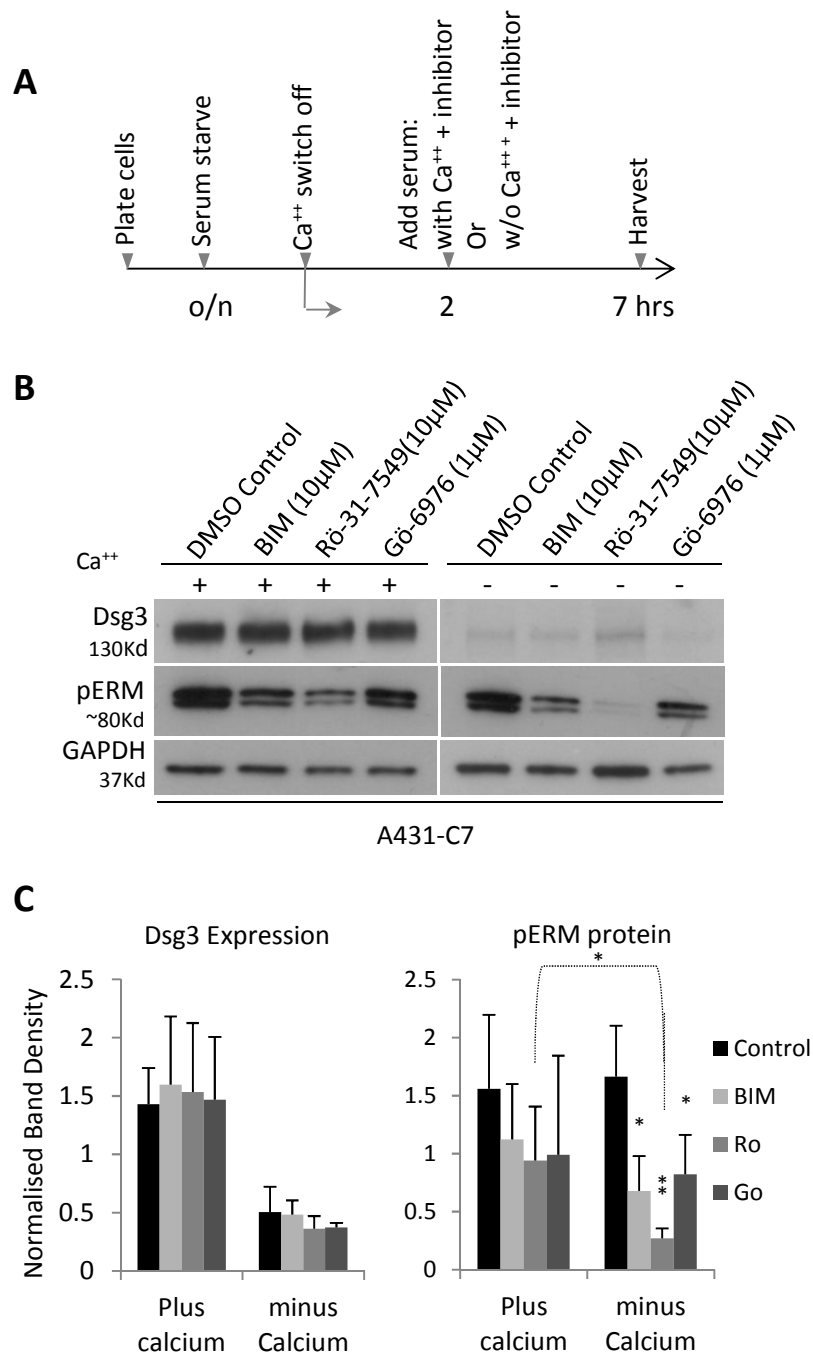


Figure 6.11 Inhibition of dependent ERM protein phosphorylation by PKC inhibition is enhanced by extracellular calcium depletion. Western blot analysis of total cell lysates of A431-C7 cells, in the presence and absence of calcium, treated with various PKC inhibitors. **(A)** Schematic for experimental time line, briefly cells were seeded at sub-confluence for 4 hrs and then serum starved overnight. Media was then replaced with calcium-free media for 2 h before addition of either media with Ca⁺⁺ and inhibitor or Ca⁺⁺ -free media together with inhibitors. **(B)** Western blots of Dsg3 and pERM, a representative of three independent experiments. GAPDH was used as a loading control here. **(C)** Quantitation of relative band density from 3 independent experiments illustrated in A & B. n=3, normalised against GAPDH (*P < 0.05, **P < 0.01)

compared to the DMSO controls. On close inspection, only the inhibition of ERM protein phosphorylation by R6 31-7549 was significantly enhanced in the absence of calcium (Figure 6.10) suggesting an additive effect. Collectively, this suggests classical PKCs are involved in Dsg3-dependent Ezrin phosphorylation (activation). However, further investigation would be required to draw definitive conclusions.

6.3 Discussion

This chapter presents data suggesting that Dsg3 acts as an upstream regulator of PKC to induce Ezrin activation at the plasma membrane. The membrane localisation of the Dsg3:Ezrin complex presented in chapter 5 together with the data presented here with regard to the colocalisation between Dsg3 and PKC suggests the likelihood of a regulatory complex of Dsg3:pPKC:Ezrin at the plasma membrane.

Regarding Dsg3 in the regulation of Ezrin phosphorylation, the gain- and loss-of-function experiments presented inconsistent results. While the gain-of-function analysis showed reproducibly a significant correlation between Dsg3 expression and ERM protein phosphorylation, Dsg3 silencing failed to show the suppression of ERM phosphorylation. This inconsistency could be attributed to constitutive activation of Ezrin in cells overexpressing Dsg3. However, using fluorescent microscopy Dsg3 knockdown appeared to significantly suppress Ezrin activity by inhibiting its function as a membrane cytoskeleton linker. Together, this suggests that Dsg3 may not directly regulate Ezrin activation but rather indirectly through a mechanism involving the recruitment of Ezrin to the plasma membrane where it is phosphorylated by other protein(s). It is also possible that a compensation mechanism by other desmosomal cadherin(s) is at play as a degree of functional redundancy has been reported in other studies (Brennan, et al., 2010, Mahoney, et al., 1999, Pan, et al., 2011). Alternatively other mechanisms of ERM activation may be responsible for the sustained ERM protein phosphorylation in cells with Dsg3 silencing.

The data presented provides strong evidence of Dsg3's ability to regulate Ezrin activity and I believe that this is achieved through a mechanism involving the recruitment of Ezrin to the plasma membrane where it is activated by PKC. In support, reports have showed induced PKC activation in response to PVIgG binding to Dsg3 in human keratinocytes (Kawasaki, et al., 2006, Kitajima, et al., 1999). In addition to the recruitment of, PKC α and to a lesser extent PKC δ , η and ζ from the cytosol to the cytoskeleton (Osada, et al., 1997). Furthermore, I have demonstrated that Dsg3-dependent ERM phosphorylation is significantly suppressed by the inhibition of PKC as well as RhoA and p38MAPK, both of which have also been reported in Dsg3 specific signalling events (Berkowitz, et al., 2005, Waschke, et al., 2006), suggesting that these molecules act in concert as downstream effectors of Dsg3. On closer inspection using

dose- and time-dependent inhibition experiments, which identified a correlation between PKC and Dsg3-dependent ERM phosphorylation, suggests that Dsg3 is sequestering PKC at the plasma membrane where it phosphorylates (activate) Ezrin. This is supported by observations using fluorescence microscopy which demonstrated colocalisation of Dsg3 and PKC at the plasma membrane which was significantly higher in Dsg3 overexpressing cells compared to the Vect Ct. With regards to the specific role of RhoA, Rac1 and p38MAPK, further studies are required to elucidate their contribution to the phosphorylation of Ezrin in this system.

Taking into consideration that PKC α has been implicated in Ezrin activation and localisation (Stapleton, et al., 2002) and Dsg3-dependent signal transduction (Kitajima, et al., 1999) the data presented here provide strong evidence of a mechanism by which Dsg3 regulates the PKC/Ezrin pathway that in turn influences actin dynamics associated cell motility. Furthermore, the data implicate the classical PKC isoforms in the activation of this pathway. PKCs in general have been implicated in regulating cell adhesion, motility and invasion, with increased activity of PKC α in stimulating cell motility and invasion (Sun and Rotenberg, 1999), novel PKC η involved in cell adhesion (Chun, et al., 1996) and spreading (Berrier, et al., 2000). PKC θ has been shown to regulate migration in endothelial cells (Tang, et al., 1997) while atypical isoforms have been implicated in the organisation of the actin cytoskeleton (Laudanna, et al., 1998, Uberall, et al., 1999). To determine which isoforms(s) of PKC are specifically involved in the Dsg3:PKC:Ezrin pathway, additional study is necessary.

How Dsg3 is able to facilitate complex formation with other non-desmosomal proteins is not known. The Mass Spec data presented in Chapter 5 identifies A-kinase anchor protein (AKAP) in the Dsg3 complex. AKAP, a scaffold protein, has been shown to interact with PKC, thus theoretically AKAP could facilitate the recruitment of PKC to the Dsg3:Ezrin complex. The ability of Dsg3 to be phosphorylated has been demonstrated previously (Aoyama and Kitajima, 1999, Kawasaki, et al., 2006), however, the exact mechanism by which this occurs remains unknown but the data presented suggests that this event could facilitate the formation of the Dsg3:Ezrin:PKC complex.

6.4 Future Work

To ascertain with certainty which PKC isoform is acting downstream of Dsg3, I propose firstly, the use of DAG inhibition in conjunction with calcium depletion using both intracellular and extracellular calcium chelators, for example BAPTA_{AM} and EGTA respectively. I would expect classical isoforms to be inhibited by both DAG inhibition and calcium depletion while novel isoforms would still function in the absence of calcium but not with DAG inhibition. Finally atypical isoforms would not be affected by DAG or Calcium depletion. Once one or more of the isoforms groups have been excluded, isoform specific antibodies could be applied to identify candidates that colocalise with Dsg3 and Ezrin. This could then be validated with the use of isoform specific siRNA or antisense oligonucleotide mediated silencing to provide a more definitive identification. On speculation PKC α is the most likely candidate as it is not only shown to activate Ezrin but also to be activated downstream of Dsg3.

What would also be of value, is to knockdown Dsg3 and examine its effect on PKC sub-cellular localisation, phosphorylation and activity compared to the Vect Ct. I would expect that knockdown would inhibit the translocation of PKC to the plasma membrane where it could potentially form a complex with Dsg3:Ezrin to facilitate Ezrin phosphorylation and subsequent activation.

Thus far, with regard to PKC the use of pPKC antibodies was used as an indication of PKC activity. Fractionation of cell lysate to yield cytosolic and particulate fractions followed by enzymatic determination of PKC activity would be a more accurate indication of PKC activity and this could be carried out in cell lines overexpressing Dsg3 compared to the vector control and knockdown of Dsg3 compared to a Scrambled control to test the hypothesis that Dsg3 recruits PKC to the plasma membrane where it is utilised as a Dsg3 effector.

Finally this study proposes that non-junctional Dsg3 exhibits extra-junctional functions. To determine which pool of Dsg3 colocalises with pPKC would further validate our proposal. I would expect to observe pPKC present in the Triton soluble fraction colocalising with non-junctional Dsg3.

Chapter 7: **Desmoglein 3 regulates the activity of transcription factor, AP-1.**

7.1 Introduction

As discussed in Chapter 1 the transcription factor AP-1 has been shown to function as a regulator of EMT and cancer progression (see section 1.4). It has been previously reported that the overexpression of Dsg3 reduces the levels of E-cadherin (Tsang, et al., 2010), the loss of which being a hallmark of EMT with the dissolution of adheren junctions (Lamouille, et al., 2014).taken together with the observation reported here and previously that the overexpression of Dsg3 does not promote adhesion but rather promotes a migratory phenotype (Tsang, et al., 2010) suggesting that Dsg3 in some way promotes EMT.

The overexpression of AP-1 components and increased activity of AP-1 up-regulate the expression of AP-1 target genes, many of which have been identified as having putative roles in tumour development and progression (Hu, et al., 1994, Kustikova, et al., 1998, Marconcini, et al., 1999). Furthermore, Ezrin is a known transcriptional target of AP-1 and has been demonstrated to promote cancer cell invasiveness through c-Fos (Jooss and Müller, 1995). I show that Dsg3 may be exerting its ability to regulate pro-invasive and migratory actin based processes through the modulation of Ezrin activity

Thus it is likely that AP-1 is also involved in Dsg3 mediated signalling to promote cancer cell migration and invasion.

This chapter outlines several lines of investigation in an effort to identify potential signalling events that may orchestrate Dsg3-mediated activation of Ezrin, cell migration and invasion.

7.2 Results

7.2.1 Desmoglein 3 enhances the phosphorylation of numerous kinases.

The initial exploration of other potential signalling mechanisms involved in Dsg3-dependent signalling in A431 cells, a human phospho-kinase array (R&D systems) was performed to gain a global perspective on the effect of Dsg3 overexpression on a 46 kinases. In this study, A431-Vect Ct and –D3 Cells were plated at sub-confluent density followed by cell lysate extraction 5 h after seeding. The array was carried out immediately according to the manufacturers specification (R&D systems). The dot blots generated from the array were analysed using ImageJ to determine the density of each duplicate which represented a phosphorylated kinase. Values for each duplicate were averaged and the background subtracted to generate an average value for each phospho-kinase. Finally, the Log₂ ratio of D3:Vect Ct was calculated which allows for the identification of 2 fold variations in kinase phosphorylation in the Dsg3 overexpressing cells with respect to the control. Treating both increases and decreases equally differential phosphorylation results in a ratio value of ≥ 1 or ≥ -1 where a log₂ ratio of 1 or -1 representing a 2-fold increase or decrease, respectively. Figure 7.1A shows the result of the array. As illustrated, a wide range of kinases exhibited increased phosphorylation in A431-D3 cells relative to the Vect Ct. Among them, Pyk-2 (red bar), STAT1-Y701 (yellow bar) and c-Jun-S63 (black bar) kinases were shown to exhibit a 2-fold increase in Dsg3 overexpressing cells. STAT1 is a member of the Signal Transducers and Activators of Transcription (STAT) family. Phosphorylation of tyrosine residue 701 induces dimerisation, nuclear translocation and DNA binding to regulate the transcription of Interferon stimulated genes (Ihle, et al., 1994). Pyk-2 is a non-receptor tyrosine kinase that mediates signals from G-protein coupled receptors in the MAP Kinase pathway (Schlaepfer, et al., 1999). As described in the introduction, c-Jun is a bZIP protein and predominant component of the AP-1 transcription factor. Phosphorylation of c-Jun at serine residue 63 increases its transcriptional activation (Smeal, et al., 1994) and has also been implicated in cancer development by up regulating genes which promote proliferation, angiogenesis and to a lesser extent invasiveness of cancer (Eferl and Wagner, 2003). In summary, this array analysis indicates that various kinases are induced by Dsg3 overexpression and may participate in the regulation of Ezrin activation and cell migration and invasion.

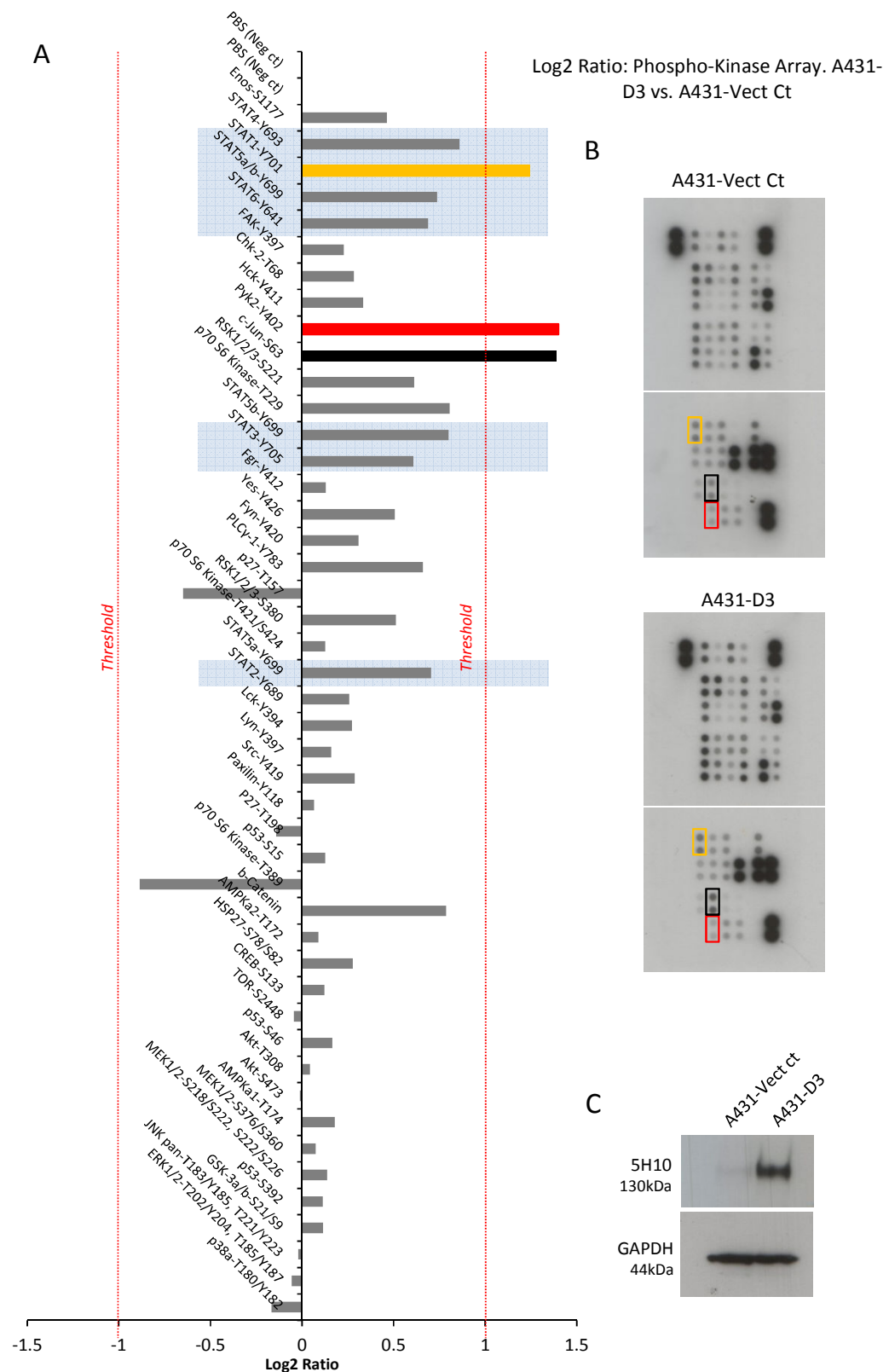


Figure 7.1 The overexpression of Dsg3 enhances the phosphorylation of a variety of kinases. A431- Vect Ct and -D3 cell lines were seeded at 50-60% density. 5 h after plating cell lysates were collected and analysed by kinase array according to the manufacturer's specification **(B)**. **(A)** Illustrated the Log2 Ratio of D3 cells vs. Vect Ct. The threshold of -1 and 1 represent a 2-fold decrease or increase Highlighted in colored bars are kinases that exhibited a 2 fold increase, STAT (yellow), Pyk2 (red) and cJun (black). The trend of increased phosphorylation of STAT kinases is highlight by blue shading. **(C)** Western blot of lysates used as an input in array analysis.

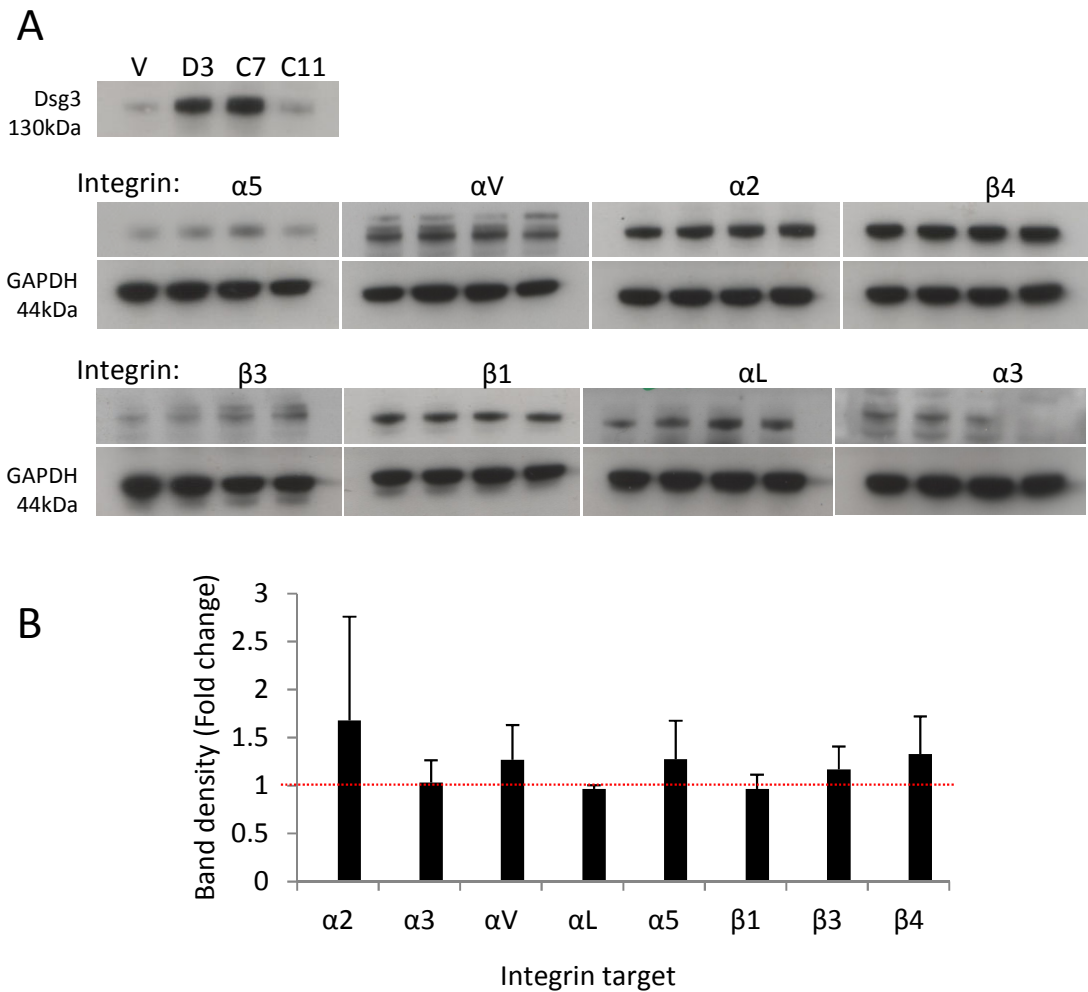


Figure 7.2 The overexpression of Dsg3 does not affect total integrin expression. (A) Western blot of total cell lysates of four A431 cell lines with high or low Dsg3 expression. **(B)** Fold change in the integrin expression in A431-D3 cells. Blots normalised against GAPDH and relative Vect control level set at 1 arbitrary unit. Data were pooled from 4 independent experiments and showed that the overexpression of Dsg3 did not significantly affect integrin protein levels except for $\alpha 2$ which showed a marginal increase (1.5-fold). The threshold of 1 indicates the Vector control expression for each protein (mean \pm SD).

7.2.2 Desmoglein 3 overexpression does not affect integrin expression.

Pyk2 has been shown to be important to the integration of signals initiated by a diverse group of extracellular stimuli, including integrin binding (Schlaepfer, et al., 1999). PYk2 has also been implicated in cell spreading and motility (Watson, et al., 2001). Integrins are heterodimeric adhesion receptors composed of an α and a β subunit that facilitate cell-extracellular matrix adhesion and also transmit signals vital to cell survival, proliferation and motility (Hynes, 2002). Integrins play a pivotal role in a range of physiological processes, including cell migration, wound healing and immune responses. Aberrant integrin signalling is involved in the development of various human diseases including cancer through their ability to influence cell

spreading (Flevaris, et al., 2007), cell motility (Holly, et al., 2000, Huttenlocher and Horwitz, 2011), invasiveness, growth and survival (Parise, et al., 2000). The observations from the phospho-kinase array (Figure 7.1) together with previous findings in cell spreading and migration suggests possible cross-talk between integrin and Dsg3 mediating signalling. To address the question whether Dsg3 is able to influence integrin expression, Western blot of total cell lysates from A431-Vect Ct, -D3, -C7 and -C11 cells was performed using a variety of integrin specific antibodies. For these experiments A431 cell lines were seeded at sub-confluence and allowed to propagate overnight prior to harvesting cell lysates. Three independent experiments were carried out comparing the effect of Dsg3 overexpression to the Vect Ct. As illustrated in Figure 7.2 the overexpression of Dsg3 appeared to have no significant effect on the expression of integrin $\alpha 5$, αV , $\alpha 3$, αL , $\beta 4$, $\beta 3$ or $\beta 1$, while integrin $\alpha 2$ showed a marginal increase compared to Vect Ct cells.

7.2.3 Desmoglein 3 does not affect STAT protein phosphorylation.

The array analysis revealed that the overexpression of Dsg3 promoted a 2-fold increase in the phosphorylation of STAT1 (Y701) and to a lesser extent the phosphorylation of STAT 4, 5a, 5b, 6 and 3 (bars highlight in Figure 7.1). The STAT protein family, of which there are 7 members, are transcription factors, which, upon JAK induced activation facilitate the transcription of the cytokine inducible genes. STAT1 facilitates the transcription of interferon-stimulated genes that are anti-proliferation, pro-apoptosis and immune surveillance (Bennett, et al., 1994, Bromberg, et al., 1996, Kaplan, et al., 1998). In this regards, it is considered to be a tumour suppressor. However, inappropriate activation of STAT1 has also been observed in a variety of malignant cells including breast cancer, head and neck squamous carcinoma and leukemia (Gouilleux-Gruart, et al., 1996, Weber-Nordt, et al., 1996), suggesting that STAT1, under specific conditions, may contribute to rather than inhibit malignant transformation (Haura, et al., 2005). STAT3, 5a/b and 6 have all been associated with cancer progression, enhancing invasiveness and a poor clinical outcome (Chen, et al., 2007, Dagvadorj, et al., 2010, Liao, et al., 2010, Merk, et al., 2011, Yu, et al., 2009). To validate the finding observed in the array and examine whether Dsg3 regulates STAT activity to promoting migration and invasion in Dsg3 overexpressing cells, Western blot analysis of total cell lysates from A431-D3 and -Vect Ct cells were performed. Blots were probed for

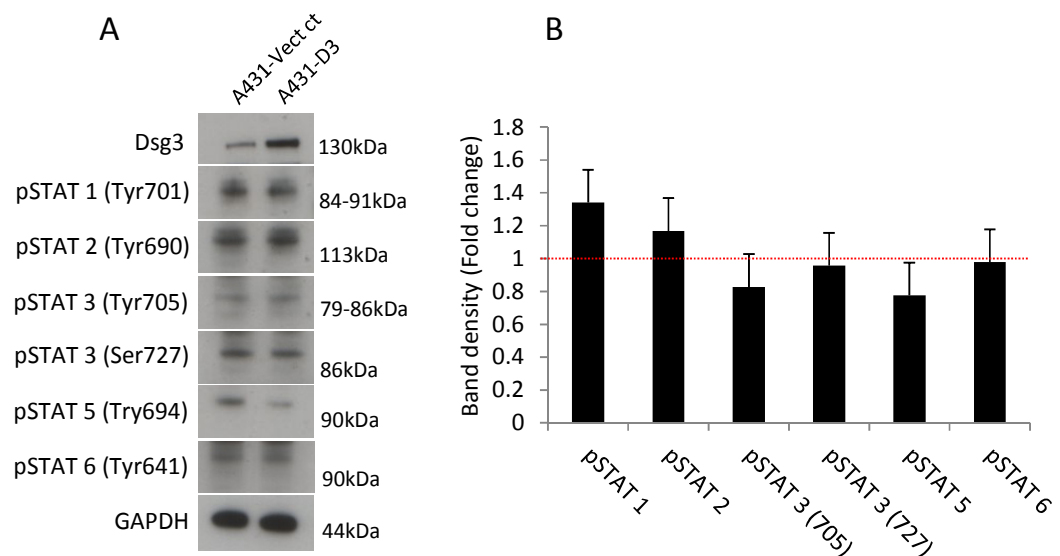


Figure 7.3 Overexpression of Dsg3 had no significant effect on the STAT phosphorylation. (A) Western blots of total lysates of A431-Vect Ct and D3 cell lines that were seeded at 50-60% density and harvested 5 h later. **(B)** Fold change in STAT phosphorylation in A431-D3 compared to vector control cells. Results were summarised from 3 independent experiments showing that overexpression of Dsg3 did not significantly affect the STAT phosphorylation. The threshold of 1 indicates the phosphorylation level of control cells. Blot densitometry: normalised against GAPDH and relative Vect control level set at 1 arbitrary unit (n= 3, mean \pm SD).

phosphorylated STAT1, 2, 3, 5, and 6 using the phospho-STAT antibody sampler kit (Cell Signalling Technology). The blots from 3 independent experiments were analysed using ImageJ to determine the band density and further in Excel to determine fold change with respect to the Vect Ct. As shown in Figure 7.3, the overexpression of Dsg3 did not appear to have any significant effect on the phosphorylation of the STAT proteins analysed.

7.2.4 Desmoglein 3 regulates c-Jun expression and phosphorylation of residues S63 and S73.

The phospho-kinase array also demonstrated an increase in c-Jun phosphorylation at serine residue S63 (pc-JunS63) in A431-D3 cells, implicating Dsg3 in the regulation of AP-1 activity. To substantiate this preliminary array finding, various methodologies including fluorescence microscopy and Western blot analyses were employed to investigate the effect of Dsg3 on c-Jun protein expression and phosphorylation at serine residues 63 (pc-JunS63) and 73 (pc-JunS73). Phosphorylation of both residues increases transcriptional activity of AP-1 (Smeal, et al., 1994). Fluorescence microscopy

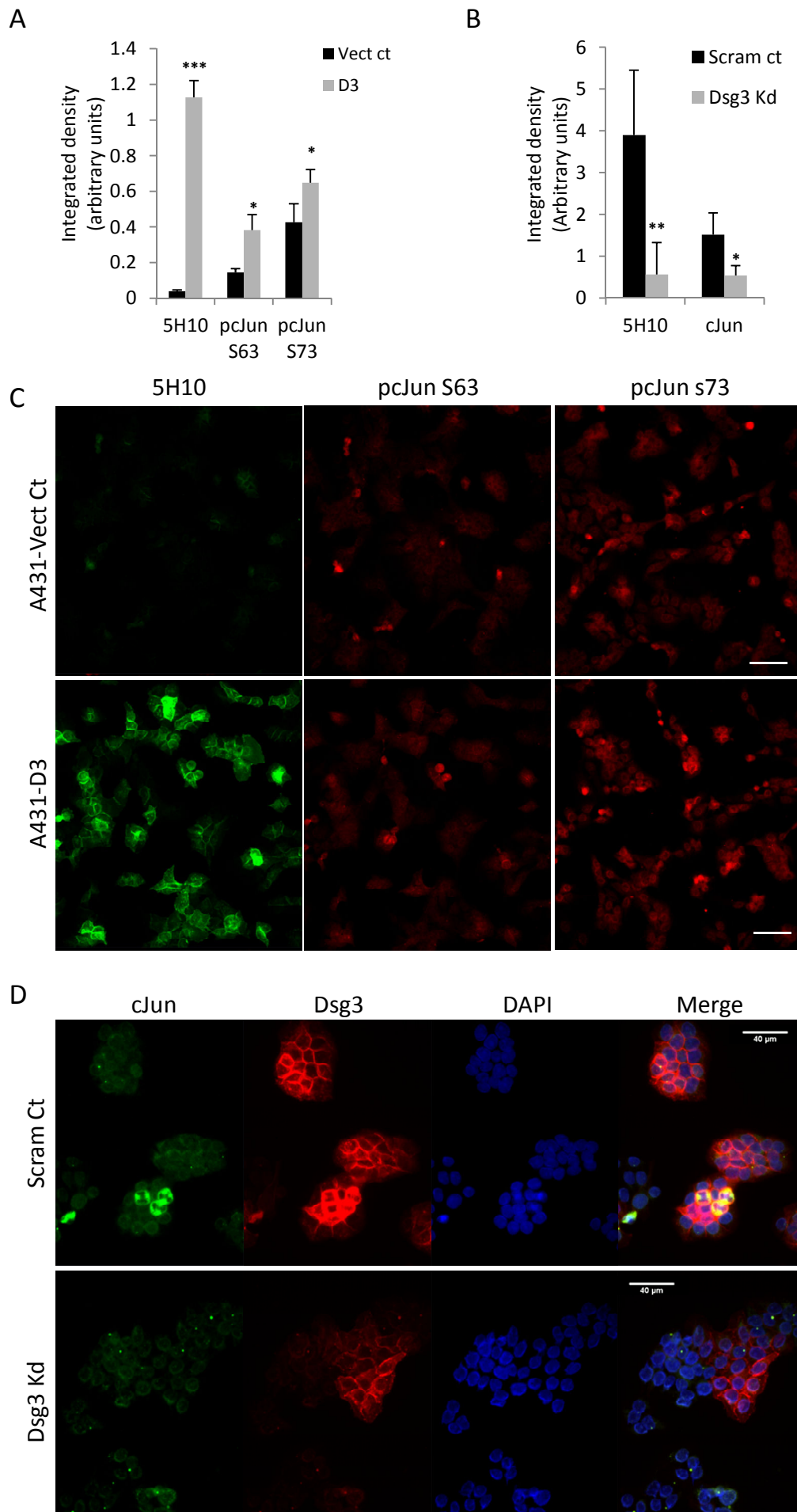


Figure 7.4 Overexpression of Dsg3 promotes cJun expression and phosphorylation at Serine 63 and 73 assessed by fluorescence microscopy analysis. Cells were seeded at 40-50% confluence and 24 h later cells were fixed with Methanol:Acetone at 1:1 and immunostained with the indicated antibodies. Three random fields were captured and images were analysed using ImageJ to determine the integrated density per cell. **(A)** The overexpression of Dsg3 showed to significantly increase the phospho-cJun S63 and S73 staining compared to vector control cells. **(B)** Knockdown of Dsg3 in A431-D3 significantly reduced the total cJun staining (n=2. **P<0.01, *P<0.05).

was performed on A431 cell lines that were seeded on coverslips 24 h prior to fixation and immunostaining. The acquired images were analysed using ImageJ to determine the integrated density per cell for each protein target. As illustrated in Figure 7.4A, a significant increase in pc-JunS63 (~2-fold) and pc-JunS73 (~0.5 fold) was identified in A431-D3 cells compared to the Vect Ct (p<0.05). Knockdown of Dsg3 in A431-D3 cells showed an opposite effect with a marked reduction in total c-Jun protein expression (Figure 7.4B). To investigate the dose-dependent nature between Dsg3 and cJun, high and low Dsg3 expressing cells, A431-C7 and -C11 respectively, were included in Western blot analysed together with A431-Vect Ct and D3 cells. As illustrated in Figure 7.5 a clear dose-dependent correlation between Dsg3 expression and the phosphorylation of c-Jun-S63 and S73 was displayed. No significant relationship was observed between total cJun and Dsg3 expression in this analysis (Figure 7.5). Taken together, the data presented here suggests that Dsg3 regulates the c-Jun phosphorylation.

It has been reported that the sub-cellular localisation of c-Jun correlates with transcriptional activity (Nateri, et al., 2004, Weiss, et al., 2003). To ascertain if modulation of Dsg3 affects the cellular localisation of c-Jun, fluorescence microscopy was performed on A431-D3 cells transfected with either scrambled control or Dsg3 specific siRNA as described previously. Nuclear staining of c-Jun in both control and Dsg3 knockdown cells was quantitatively analysed using ImageJ to generate cross-sectional nuclear staining profiles. The graphical presentation of the staining profiles shown in Figure 7.6 illustrated that compared to control cells, knockdown of Dsg3 in A431-D3 cells reduced the nuclear localisation of c-Jun, observed as a reduction in the integrated density (mean grey value) within the nucleus. Collectively, these studies suggest that Dsg3 regulates c-Jun phosphorylation (activation) at S63 and S73 which is essential for the dimerisation and AP-1 transcription activity in the nucleus.

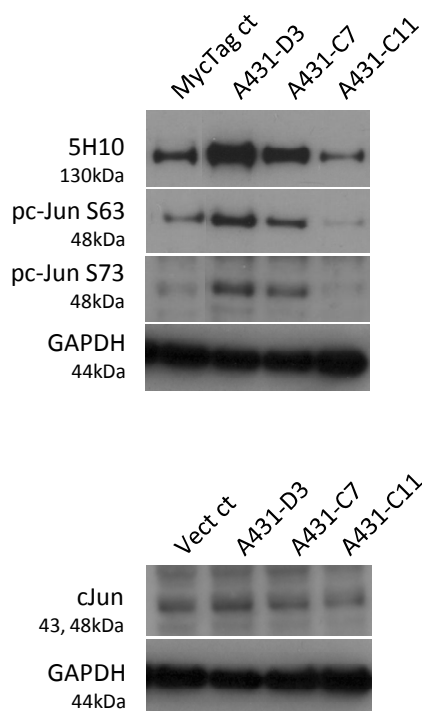


Figure 7.5 Overexpression of Dsg3 enhances the phosphorylation of cJun-S63 and S73 but not total cJun. Western blotting analysis of A431 cell lines expressing high (D3, C7) and low (Myc tag Ct, C11) Dsg3. Total cell lysates were harvested 5 h after cell seeding at 40-50% confluence. An increased phosphorylation of cJun-S63 and S73 was detectable in both D3 and C7 compared to MycTag Ct (expressing endogenous Dsg3) and C11 cells, indicating a clear correlation between the phosphorylation of cJun-S63 and S73 and the level of Dsg3 expression. Dsg3 expression does not appear to affect total cJun expression (n = 3).

7.2.5 Desmoglein 3 promotes AP-1 transcriptional activity.

Thus far, the data suggests that Dsg3 regulates the phosphorylation of c-Jun at S63 and S73, a prerequisite for transcriptional activity. To evaluate whether the increased cJun phosphorylation confers increased AP-1 activity in A431 cells, a Luciferase reporter assay was used. This assay works on the principle that the expression of target genes can be used as an assessment of transcriptional activity. Based on this principle, the Luciferase enzyme gene is inserted downstream of the AP-1 promoter resulting in the transcription of the Luciferase enzyme. The level of Luciferase transcription can be measured directly by the addition of the Luciferase enzyme substrate. In the presence of Luciferase enzyme the addition of substrate results in luminescence, the amount of which is directly proportional to the level of AP-1 transcriptional activity.

The Luciferase reporter assay was performed using A431-parental cells which were co-transfected with increasing concentrations (0, 0.25, 0.1 and 1 µg) of pBABE-hDsg3.myc, supplemented with pBABE-GFP to make the total concentration of cDNA constant (*i.e.* 1 µg per well), together with either wild type AP-1:luciferase construct or vector control construct (1 µg per well) which served as a negative control. As illustrated in Figure 7.7A, the transient overexpression of Dsg3 in A431 cells at increasing concentrations prompted a correlated increase in luciferase activity observed as a

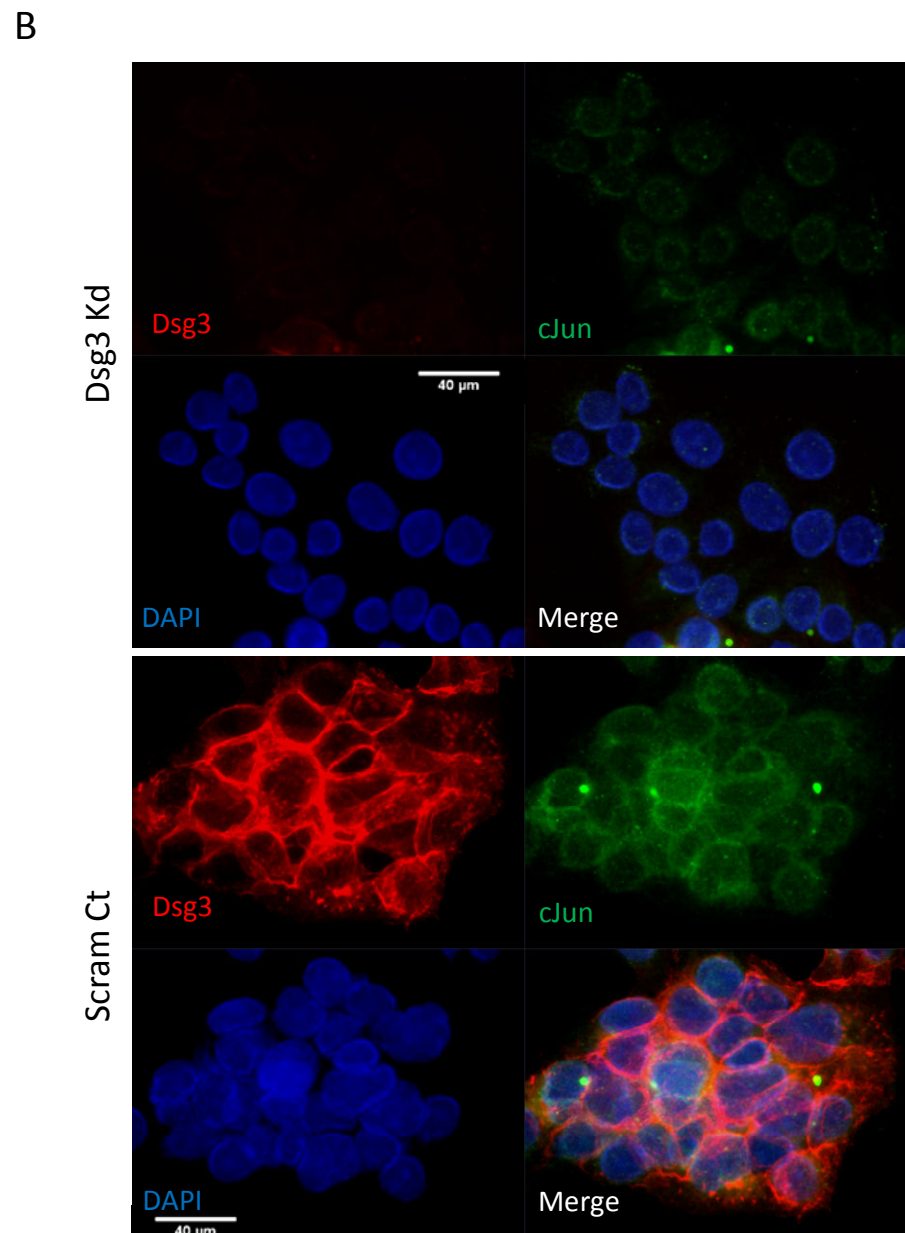
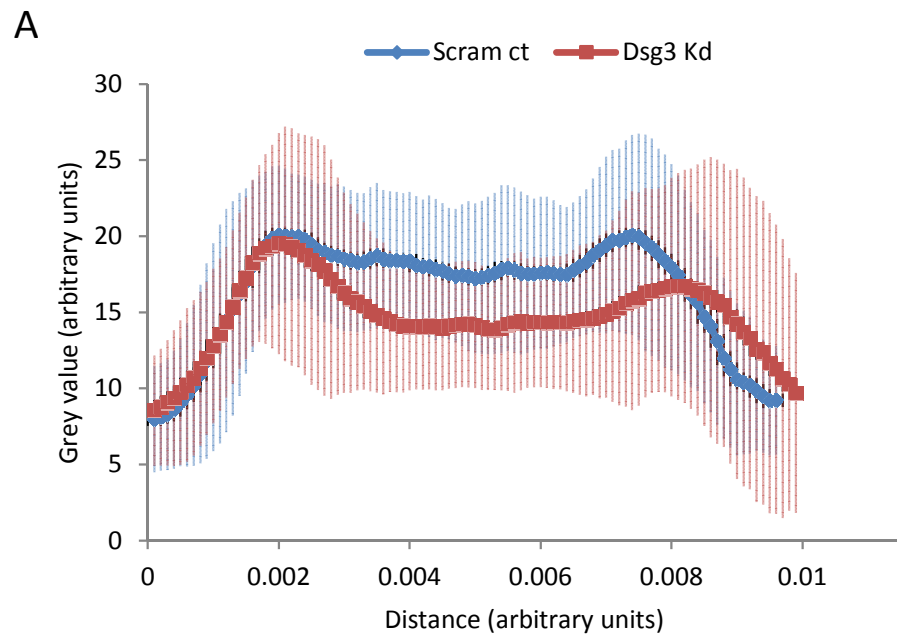


Figure 7.6 Knockdown of Dsg3 affected the nuclear localisation of cJun. A431-D3 cell lines treated with either Dsg3 specific or scrambled control siRNA at a final concentration of 50 nM for 48 h before fixation and immunostained with total cJun antibody. The immunofluorescence profiles across each nucleus of >60 cells were analysed in both scrambled Ct and knockdown cells and showed that knockdown of Dsg3 reduced cJun nuclear staining. Scale bar 40 μ .

significant increase in luminescence. To further validate this finding, a knockdown study was performed in A431-D3 cells. Cells were transiently transfected with Dsg3 specific or scrambled Ct siRNA. The following day, cells with or without Dsg3 knockdown were subjected to transfection with either wild type AP-1 or vector:*Luciferase* construct. The Luciferase assay was performed a further 24 h later. As shown in Figure 7.7B, Dsg3 silencing in A431-D3 cells significantly reduced (~4-fold) Luciferase activity compared to the scrambled Ct. Collectively, these results indicate that Dsg3 regulates the AP-1 transcriptional activity in a dose-dependent manner. To ascertain that the Dsg3-dependent AP-1 activity was not restricted to A431 cells but rather a universal mechanism, the AP-1 response to the transient overexpression of Dsg3 was also analysed in MDCK and Cos-1 cell lines (courtesy of MSc student, Suzan Hama). Cos-1 and MDCK cells were subjected to co-transfection as described above and the luciferase activities were determined 48 h later. As shown in Figure 7.8, a significant increase (~4-fold) in luciferase activity was consistently observed in cells transfected with hDsg3.myc but not GFP control, indicating that Dsg3 is also able to regulate AP-1 transcriptional activity in other cell types. Taken together, these findings corroborate the above finding and strongly suggest that the Dsg3-dependent regulation of cJun:AP-1 activity is not restricted to A431 cells but appears to be a universal mechanism.

7.2.6 Desmoglein 3-dependent AP-1 activity is abrogated by JNK, PKC, p38 and Rac1 inhibition.

In an attempt to identify the downstream effectors of Dsg3 which leads to AP-1 activation, the Luciferase assay was carried out in conjunction with various inhibitors that target a range of signalling pathways. In this case, 24 h after co-transfection, cells were re-seeded and allowed to adhere for 6 h. Media was then changed to serum free media, and cells were subjected to serum starvation overnight. The following day, inhibitors diluted in growth medium (DMEM supplemented with 10% serum) were added to cultures for 4 h before proceeding with the Luciferase assay (assayed 48 h

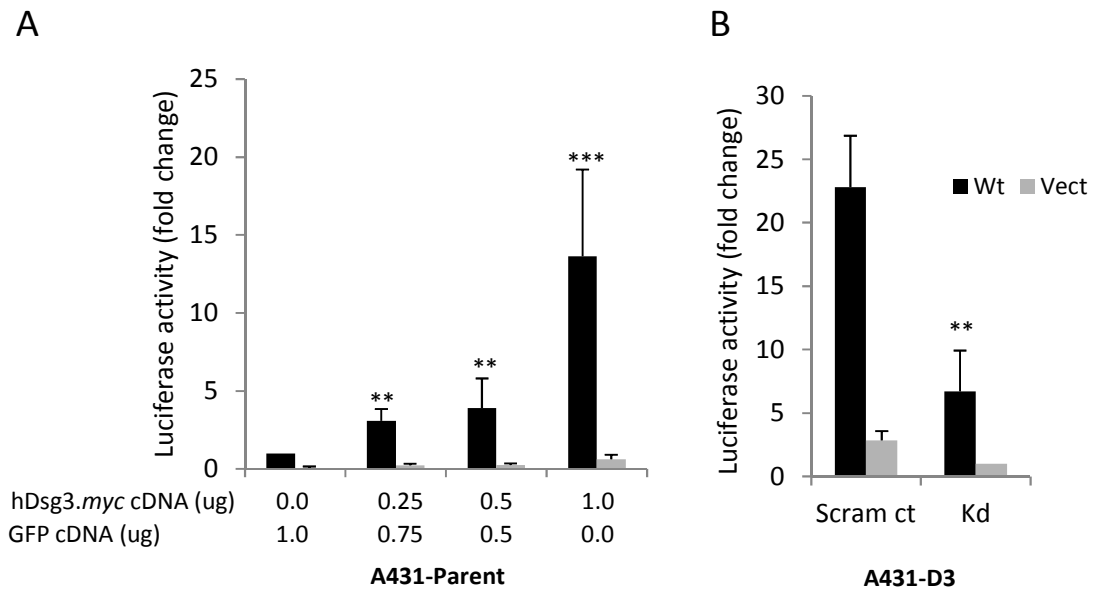


Figure 7.7 Overexpression of Dsg3 promotes AP-1 transcriptional activity. (A) Luciferase reporter assay demonstrates a significant increase in the AP-1 transcriptional activity in a dose-dependent manner in A431 parental cells co-transfected with pBABE-hDsg3.myc and AP-1:Luciferase construct (black bars) but not with pBABE-hDsg3.myc and Vector:Luciferase construct (grey bar). The total amount of cDNA was supplemented by pBABE-GFP (as stated) and 1ug of each construct was used so a total of 2ug of constructs were used in each well (n=3, *** $P < 0.001$, ** $P < 0.01$). **(B)** Silencing of Dsg3 in A431-D3 cell line significantly reduces the AP-1 transcriptional activity. Cells were treated with either Dsg3 or scrambled control siRNA at a concentration of 50nM for 24 h prior to transfection with 1ug AP-1:Luciferase construct (black bar) or Vector:Luciferase construct (grey bar). After further culture for 24 h cell lysates were harvested and assayed for Luciferase activity. The representative result is shown (n=3, ** $P < 0.01$).

after transfection). The concentrations of inhibitors used in this experiment are as described previously (Table 2.2). As indicated in Figure 7.9, the Dsg3-dependent activation of AP-1 appeared to be abrogated by inhibition of JNK, PKC, p38, Src and Rac-1, respectively (courtesy of a Master student Sazan Hama), suggesting their involvement in the Dsg3 mediated AP-1 activation.

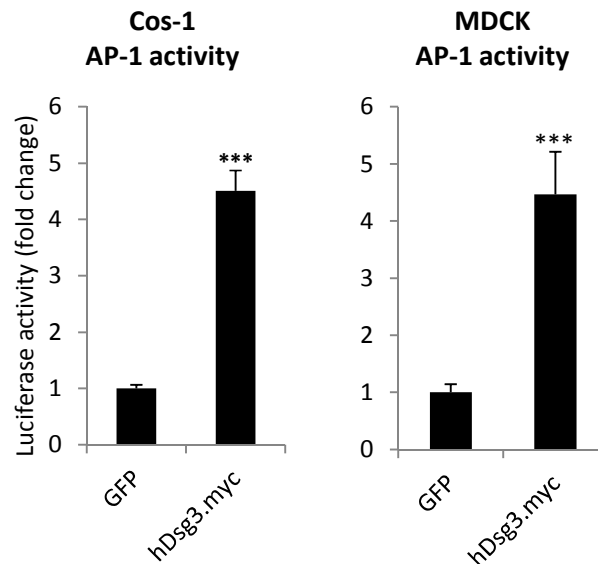


Figure 7.8 Dsg3 regulates the AP-1 transcriptional activity in Cos-1 and MDCK cells. Cos-1 and MDCK cell lines were co-transfected with either GFP or hDsg3.myc construct in conjunction with wild type AP-1:Luciferase construct. 48 h later, the Luciferase assay was carried out. As shown, the overexpression of Dsg3 resulted in increased Luciferase activity (n=3 per cell line, *** P< 0.001; courtesy of Suzan Hama).

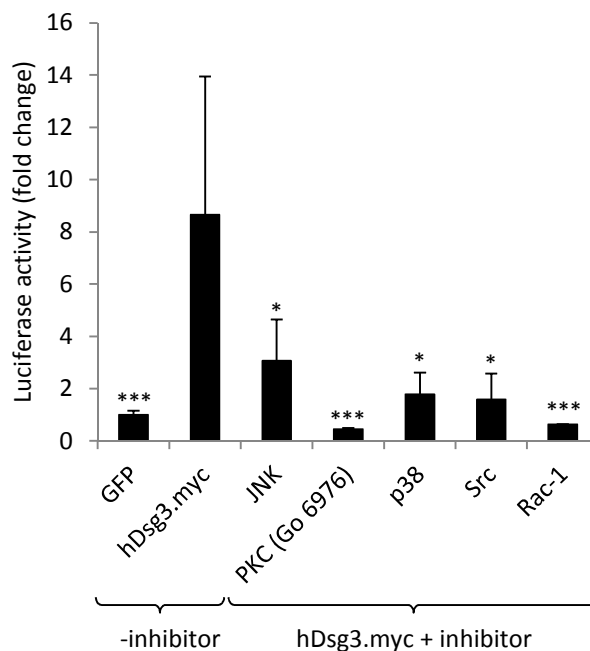


Figure 7.9 Inhibition of PKC, p38, Src and Rac-1 abrogated Dsg3-dependent activation of AP-1 transcription. Cos-1 cells were co-transfected with either GFP or hDsg3.myc and WT AP-1 targeting luciferase construct. Twenty-four h following transfection, cells were serum starved overnight. Forty-eight h after transfection, cells were treated with the indicated inhibitors diluted in DMEM plus 10% serum for 4 h. Total cell lysates were collected and assayed for luciferase activity (n=3) courtesy of Suzan Hama, Mean luciferase activity \pm SD, all samples were compared to that with Dsg3 overexpression, ***P<0.001, *P<0.05).

7.3 Discussion

In summary, the data presented in this chapter demonstrate a novel function of Dsg3 as an upstream regulator of AP-1 transcriptional activity through the modulation of cJun phosphorylation.

The ability of Dsg3 to act as a cell surface receptor for various intracellular signalling has been established through studies employing Dsg3 autoantibody which upon binding provokes the activation of p38 and PKC as downstream effectors of Dsg3-dependent actin reorganisation (Kawasaki, et al., 2006). This finding is in agreement with the data presented in this study, that provides evidence that Dsg3 regulates c-Jun:AP-1 activity through JNK, p38 and PKC. The activation of PKC by phorbol esters or diacylglycerol has been reported to induce at least two MAPK (mitogen-activated protein kinase) pathways, the ERK (extracellular signal-regulated kinase) pathway and JNK (c-Jun N-terminal kinases) pathway (Lang, et al., 2004), with the induction by PKC increasing AP-1 activity through the modulation of c-Jun, c-Fos and ATF2 (Boyle, et al., 1991).

A recent study by Chen *et al* (2013) has demonstrated that *DSG3* silencing increased the translocation of plakoglobin (Pg) to the nucleus where it interacts with the TCF/LEF transcription factor and inhibits its transcriptional activity to suppress tumour growth and invasion (Chen, et al., 2013). Previous work by Dr Wan has shown that the overexpression of Dsg3 in A431 cells promotes an increase in tyrosine phosphorylation of β -catenin (Tsang, et al., 2010) In support Figure 7.1 illustrates an increase in β -catenin phosphorylation although below the cut off threshold (2-fold). Tyrosine phosphorylation of β -catenin at residue 142 has been identified to increase its dissociation from the E-cadherin adhesion complex and enhance its association with the LEF/TCF cofactor, BCL9 as a result of increasing β -catenin transcriptional activity (Brembeck, et al., 2004). Additionally, increased β -catenin activity (2~fold) was detected in A431-D3 cells compared to the Vect Ct (unpublished data). It has also been reported that c-Jun interacts with TCF4 to form a tertiary complex containing c-Jun, TCF4 and β -catenin which was shown to interact with the c-Jun promoter thus conferring transcription activity, in a JNK-dependent manner, in intestinal tumourigenesis (Nateri, et al., 2005). Thus it is likely that the phosphorylation of β -catenin in Dsg3 overexpressing cells also contributes to the activation of cJun.

Together, these reports are consistent with the proposal that Dsg3 acts as an upstream regulator of JNK/cJun pathway to promote cancer cell migration and invasion.

The relationship, or lack of, between Dsg3 and total c-Jun proves to be of less significance compared to its phosphorylation suggesting that Dsg3 regulates the activity of c-Jun:AP-1 through post translational modification rather than by regulating its expression. Phosphorylation of c-Jun at N-terminal S63 and S73 mediated by JNK is considered to be the most important regulatory phospho-sites by increasing the transcriptional activation of AP-1 (Dérjard, et al., 1994) as both a c-Jun homodimer (Pulverer, et al., 1991, Smeal, et al., 1991) or heterodimer (Deng and Karin, 1994). The phosphorylation of these sites has also been implicated to reduce ubiquitination and subsequent degradation of c-Jun, thus increasing its stability (Musti, et al., 1997).

As discussed in Chapter 1, AP-1 dimeric composition determines the genes that are regulated. The data presented thus far implicates c-Jun as a downstream effector of Dsg3. However, what remains unknown is whether the Dsg3 mediated AP-1 activity is facilitated by c-Jun homodimers or heterodimers. Both c-Jun homodimers and c-Jun:c-Fos heterodimers preferentially bind the TRE (Smeal, et al., 1989), however, the stability and DNA binding affinity of the heterodimer is significantly higher than that of homodimers (Rauscher, et al., 1988). As a result, when co-expressed the heterodimeric combination results in more efficient activation of AP-1 response genes (Chiu, et al., 1988, Rauscher, et al., 1988, Smeal, et al., 1989). Based on this observation and that provided by Cavigelli *et al* who demonstrates that MAPKs, JNK and ERK are capable of activating Elk-1:TCF to induce c-Fos and ATF expression (Cavigelli, et al., 1995) one would speculate that Dsg3 regulates heterodimeric AP-1 rather than c-Jun homodimers. However, it remains undefined whether overexpression of Dsg3 acts also cFos. It is possible that activation of cFos also occurs in Dsg3 overexpressing cells that in turn contributing to the enhanced migratory phenotype of these cells.

With regards to Integrin expression, the data presented in this Chapter suggests that Dsg3 overexpression has no significant effect on integrin protein expression. However, it cannot be ruled out that the integrin activity may vary in relation to the Dsg3 expression which warrants further study. In addition, the culture condition and cell harvesting time point may not have been optimal to identify differential integrin profiles. Furthermore, the phosphorylation status of integrins could also be of

importance as it has been shown that the phosphorylation of β subunit of integrin regulates integrin activation.

While this aspect of this study are still in its infancy, the data presented in this Chapter provides strong evidence that Dsg3 regulates c-Jun:AP-1 transcriptional activity to promote cancer cell migration and invasion, collectively, providing a valuable link between novel non-junction Dsg3 function and an established mechanism of c-Jun:AP-1 activation in cancer development.

7.4 Future work

The work carried out in this chapter provide the basis for additional studies to determine the downstream effectors of Dsg3 which lead to the activation of JNK and the dimeric composition of c-Jun:AP-1 which would ultimately uncover the AP-1 specific transcriptional array of target genes induced by Dsg3 to promote migration and invasion. It would also be of interest to examine the effect of Dsg3 expression of cFos activity.

To confirm that Dsg3-dependent activation of AP-1 activity infers migration and invasion, functional assays of stable Dsg3 cell lines (A431-D3 or C7) transfected with either c-Jun specific or Scrambled siRNA could be performed. I would expect to observe inhibited migration and invasion in c-Jun silenced cells compared to the Scrambled control and Vect Ct cells.

Preliminary inhibitor study findings support PKC's role as a downstream effector of Dsg3 in regulation of AP-1 activity but also implicates p38, rac1 and Src downstream of Dsg3 with their inhibition resulting in a significant reduction in AP-1 activity (Figure 7.9) as well as ezrin activation (Figure 6.7). Further to this study, the inhibition of Dsg3 and the effect on the activity, location and abundance of these proteins will validate their contribution to Dsg3 dependent signalling. Finally functional assays with respect to invasion with p38, Rac1 and Src silencing would further substantiate their roles as downstream effectors in Dsg3-dependent cell invasion.

As discussed, phosphorylation of Integrins, particularly the β subunit is a regulatory event and one that has also been implicated in oncogenesis. It would be interesting to look at the phospho-status of β integrins in response to Dsg3 expression. I would expect to see an increase in phosphorylation, which would result in an increased affinity of DoK1 and subsequent reduction in Cell-Extracellular matrix adhesion as a means to promote migration and invasion (Tsang, et al., 2012).

Chapter 8: **Concluding Discussion and Future Perspective.**

8.1 Concluding Discussion

The development and progression of malignancy is a highly complex multistage process (Woodhouse, et al., 1997), which alters a cell's biology in its entirety to fundamentally affecting the way the cell interacts and responds to its surrounding microenvironment. The leading cause of cancer related fatalities, with > 90% of cancer deaths, is attributed to metastasis (Coghlin and Murray, 2010, Gupta and Massagué, 2006, Steeg, 2006). What is becoming clear is the need to expand the development of anti-metastatic therapies, the progress of which is currently limited by our understanding of invasion and metastasis in cancer cell biology. The identification of metastatic biomarkers is most definitely the first step in the treatment of disseminated disease, however to successfully translate them into therapeutic targets requires greater comprehension of their roles in the molecular mechanisms of these processes.

The aim of this study was to examine the role of Dsg3 in the regulation of cancer cell morphology, motility and invasion. The results provided in this thesis demonstrate a positive role of Dsg3 in cancer progression and metastasis in line with recent reports in the literature. As illustrated in Figure 8.1 my study shows: 1) the overexpression of Dsg3 promotes membrane protrusions and cell spreading and increases migration and

invasion; 2) non-junctional Dsg3 colocalises and forms a molecular complex with Ezrin predominantly at the basolateral domains of the plasma

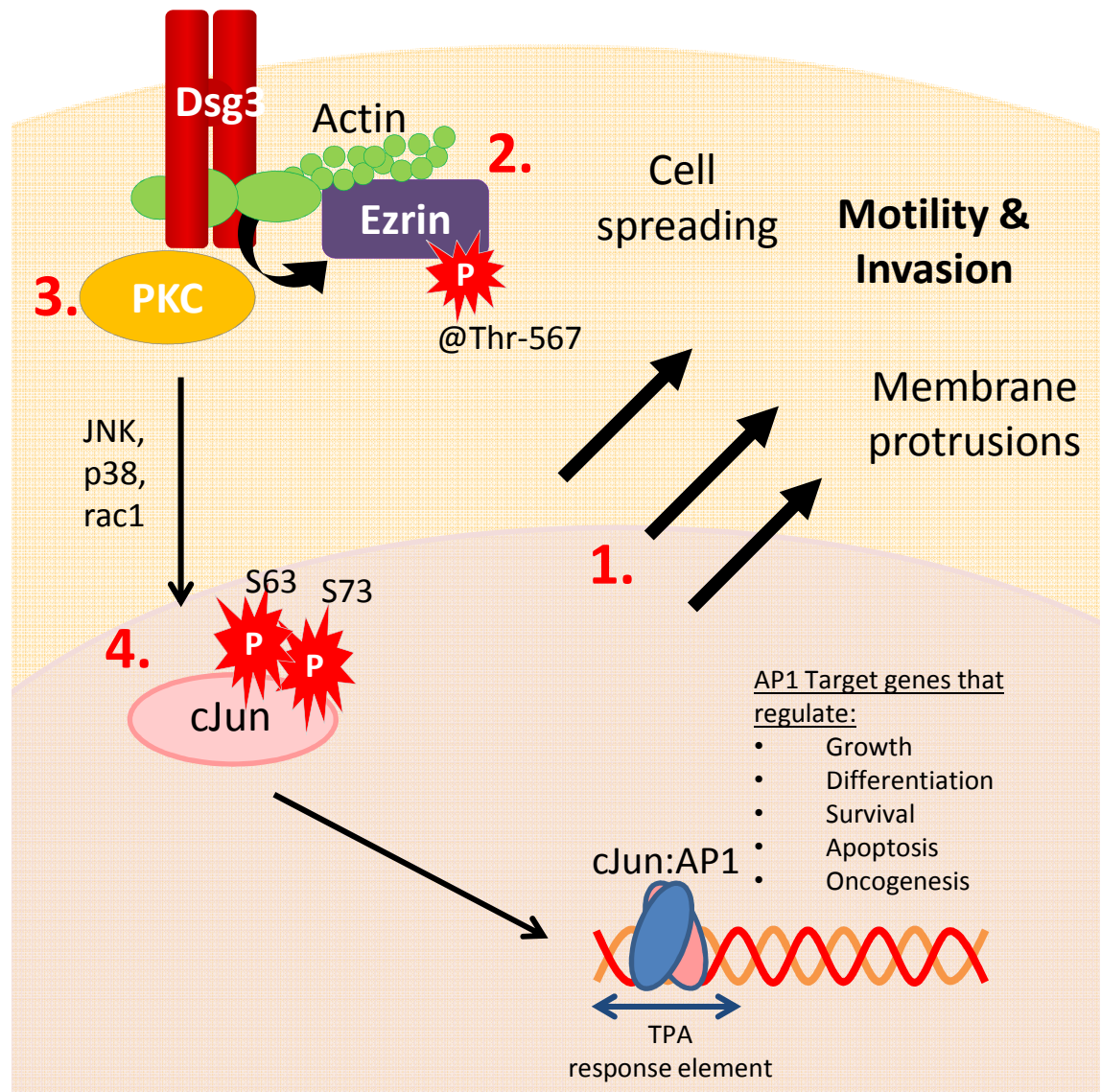


Figure 8.1. Desmoglein 3 regulates cancer cell migration and invasion through AP-1 and PKC-dependent Ezrin activation. Illustrated is a schematic of the proposed mechanisms by which Dsg3 promotes cell migration and invasion in A431 cells. Dsg3 recruits Ezrin via actin to the plasma membrane where Ezrin is activated through a PKC-dependent mechanism. Furthermore, Dsg3 acts as an upstream regulator of cJun S63 and S73 phosphorylation to promote cJun:AP-1 transcriptional activity.

membrane; 3) Dsg3 regulates Ezrin activity at the plasma membrane, likely in a PKC-dependent manner; and finally, 4) Dsg3 also acts as an upstream regulator of cJun:AP-1 transcriptional activity. In conclusion, my study suggests that Dsg3 promotes cancer invasion and metastasis through the mechanisms of PKC-dependent Ezrin and cJun:AP-1 activation and may serve as a potential drug target for the treatment of cancer.

Phenotype of Dsg3 overexpressing cells

Genetic profiling of numerous cancers has identified the aberrant overexpression of Dsg3 which appears to correlate with disease progression and metastasis as discussed previously. I provide evidence that Dsg3 regulates cell morphology, migration and invasion. These observations are consistent with reports by Tsang *et al* who demonstrated enhanced membrane protrusions and cell migration in Dsg3 overexpressing A431 cells (Tsang, et al., 2012). In support independent studies using animal models to examine the effect of differential expression of Dsgs in the epidermis (Elias, et al., 2001, Merritt, et al., 2002) showed that the specific expression patterns of Dsgs have a regulatory role in tissue morphogenesis and homeostasis. These studies demonstrated, through targeted mis-expression of Dsg3 in the epidermis, altered cell morphology, proliferation and differentiation. Furthermore a Dsg3 knockdown study in head and neck cancer cell lines showed that upregulation of Dsg3 elicits increased migration and invasion both *in vitro* and *in vivo* (Chen, et al., 2007).

The ability of Dsg3 to regulate actin based cellular processes was recently demonstrated in a study by Tsang *et al*, which showed that non-junctional Dsg3 (Triton-soluble) co-immunoprecipitates with actin and regulates the activity of Rho GTPases, Rac1 and Cdc42, to modulate actin organisation and membrane dynamics (Tsang, et al., 2012). Furthermore, studies based on PV which employ the use of anti-Dsg3 auto-antibodies also demonstrate Dsg3-dependent regulation of the actin cytoskeleton as a fundamental mechanism in the pathogenesis of the disease (Gliem, et al., 2010). Consistent with previous reports, my observations provide compelling evidence towards Dsg3 function in the regulation of cell morphology, migration and invasion. I believe that Dsg3 is capable of regulating actin indirectly through the modulation of Ezrin activity.

Desmoglein 3 associates with and regulates Ezrin activity

Ezrin's ability to regulate actin based processes, including cell polarity (Zhu, et al., 2010), lamellipodia and filopodia formation (Baumgartner, et al., 2006, Gandy, et al., 2013), cell spreading (Mak, et al., 2012) and migration (Meng, et al., 2010, Ng, et al., 2001) has been well established in the literature. So too is the aberrant regulation of Ezrin in cancer with its altered expression, phosphorylation and sub-cellular localisation shown to correlate with enhanced migration, invasion and metastasis (Chen, et al., 2011,

Chuan, et al., 2006, Donatello, et al., 2012, Elliott, et al., 2005, Heiska, et al., 2011, Ma and Jiang, 2013, Meng, et al., 2010, Xie, et al., 2011). The specific mechanisms by which Ezrin confers this vast array of functions in health and disease are still being elucidated. However, as currently understood involves: the organisation of specialised membrane domains that bring ligands, receptors and effector molecules into close proximity (Lamb, et al., 1997, Saotome, et al., 2004, Zhu, et al., 2010); and secondly, the initiation of signalling pathways. The specific pathways that are activated by Ezrin and subsequent cellular response is dependent on which kinase facilitates its phosphorylation and the specific phosphorylation sites (Clucas and Valderrama, 2014). For example, phosphorylation of Ezrin Thr567 by PKC α promotes cell migration (Ng, et al., 2001), while phosphorylation by Akt is associated with NHE3 translocation and activation that leads to cytoplasmic localisation in intestinal epithelial cells (Shiue, et al., 2005) and phosphorylation by Rho-kinase confers Ezrin to function as an effector of RhoA (Clucas and Valderrama, 2014). Furthermore the EGFR-dependent phosphorylation of Ezrin at Try145 and Try353 promote cell proliferation and lamellipodia formation (Gandy, et al., 2013, Krieg and Hunter, 1992), while phosphorylation of Try145 by Src promotes cell spreading (Mak, et al., 2012); Finally Ezrin's functional array is also conferred by its vast and expanding repertoire of proteins that Ezrin can associate with through either directly binding to integral membrane proteins, including CD44, EGFR and ICAM1, or through membrane associated proteins such as scaffold proteins, EBP50 and PDZK₁ which themselves have two and four PDZ domains, respectively, (LaLonde, et al., 2010, Morales, et al., 2004).

Precisely how Ezrin participates in cancer cell migration and invasion is not fully understood, however the overexpression of Ezrin rather than its phosphorylation is the most common aberration reported in cancer. I did not observe an increase in Ezrin expression in response to Dsg3 overexpression but rather its hyper-phosphorylation and altered cellular localisation. I show increased phosphorylation of Ezrin Thr567 and its colocalisation with Dsg3 at basolateral domains of plasma membrane in A431 Dsg3 overexpressing lines compared to control cells. The phosphorylation of Ezrin Thr567 is essential to activation (Fievet, et al., 2004, Matsui, et al., 1998) and associated with the establishment of cell polarity (Casaletto, et al., 2011, Zhou, et al., 2005, Zhu, et al., 2010), filopodia and lamellipodia formation (Baumgartner, et al., 2006, Gandy, et al.,

2013, Krieg and Hunter, 1992), changes in cell morphology (Belkina, et al., 2009) and migration (Ng, et al., 2001). In cancer, phosphorylation of Ezrin Thr567 has been implicated in tumour metastasis, with hyper-phosphorylation associated with metastasis of hepatocellular carcinoma (Chen, et al., 2011), osteosarcoma (Ren, et al., 2009) and colorectal cancer (Leiphrahpam, et al., 2014). In all these studies, the increased phosphorylation of Ezrin T567 is seen to be accompanied by increased expression levels, which is contrary to what I observed in this study. This discrepancy could be attributed to increased turnover of Ezrin proteins or the dynamic regulation of Ezrin expression (Ren, et al., 2009) which may not be evident in endpoint Western blot and fluorescence microscopy analysis carried out in this study.

Mislocalisation of ERM proteins is believed to interfere with complex formation and subsequent signalling pathways which could aid tumour progression (Arpin, et al., 2011). Abnormal distribution of Ezrin has also been shown in invasive breast and lung cancer cells. In both cases, the re-localisation from apical regions to motile projections and cytoplasm were reported (Li, et al., 2012, Sarrió, et al., 2006). This non-polar (apical-basal axis) redistribution of Ezrin in invasive cancer cells suggests that its function in cancer mechanics is not only modulated by its activation but also by its temporal and spatial localisation. With this in mind, it seems plausible that Dsg3 regulates the activity of Ezrin by facilitating its activation through PKC and targeting Ezrin to specific cellular domains by recruiting Ezrin to the basolateral domain of the plasma membrane to alter cell polarity and promote the formation of locomotion and invasive membrane structures. This proposal is in part supported by a recent study by Tsang *et al* that demonstrated the ability of Dsg3 to regulate cell polarity in normal epithelial cells (Tsang, et al., 2012). Another study showed increased localisation of phosphorylated Ezrin T567 at the plasma membrane of invadopodia of invasive breast tumour cells with the phosphorylation of Ezrin inducing invadopodia-dependent ECM proteolysis and invasion through a mechanism involving $\beta 1$ -integrin and NHE1 (Na^+/H^+ exchanger type 1) (Antelmi, et al., 2013).

To further support Dsg3 ability to regulate actin based processes through Ezrin, I show reproducibly that Dsg3 not only colocalises but also forms a molecular complex with Ezrin, at the plasma membrane. I have provided evidence by Mass spectrometry that this is most likely a multi-protein complex that includes scaffold proteins as well as

regulators of actin such as ankyrin and RhoGEF proteins. The presence of these proteins would theoretically support not only multi-protein complex formation but also facilitate the regulation of actin dynamics and Dsg3-dependent Ezrin phosphorylation. Taken together, my findings suggest that Dsg3 promotes cancer cell migration and invasion through functionally interacting with Ezrin, among others, at the plasma membrane.

Desmoglein 3 regulates the AP-1 activity

AP-1 is regarded as central to the regulation of the multigenic invasion programme required for transformation, development and progression of cancer (Dong, et al., 1997, Malliri, et al., 1998, Saez, et al., 1995, Young, et al., 1999, Yuspa, 1998). The aberrant regulation of AP-1 results in the inappropriate expression of genes that promote or suppress tumour development (Eferl and Wagner, 2003). With respect to the pro-oncogenic role of AP-1, the target gene repertoire which confers proliferation, inhibition of apoptosis, invasiveness and angiogenesis is extensive (Eferl and Wagner, 2003). With respect to migration and invasion, the regulation of the actin cytoskeleton is paramount and AP-1 target genes which confer these activities include Mts-1, CD44, stromelysin 1, Ezrin and uPAR (Hennigan, et al., 1994, Jooss and Müller, 1995, Lamb, et al., 1997, Lamb, et al., 1997) which are all implicated in cytoskeletal organisation, cell motility and remodelling of the extracellular matrix. In support, studies have shown that AP-1 is required for the activation of Rho-GTPases, localisation of CD44 and Ezrin to membrane ruffles, cytoskeleton rearrangement, motility and invasion in A431 cells (Malliri, et al., 1998, Stapleton, et al., 2002). Collectively, the findings from these reports are consistent with the phenotype that we have observed in Dsg3 overexpressing A431 cells and the involvement of AP-1 activation. I have shown that Dsg3 acts as an upstream regulator of AP-1 transcriptional activity through a mechanism of cJun phosphorylation that attributes, at least in part, to enhanced cell migration and invasion.

Plakoglobin (Pg) interacts directly with the cytoplasmic tail of Dsg3 (Chen, et al., 2013, Garrod, et al., 2002, Green and Gaudry, 2000, Yin and Green, 2004). A recent study by Chen *et al.* presented evidence that Dsg3 silencing facilitates cancer cell growth and invasion through the DSG3-Pg-TCF/LEF-Myc/Cyclin D1/MMP signalling pathway (Chen, et al., 2013). The TCF/LEF transcription factors have also been implicated as

downstream effectors of Dsg3 in the pathogenesis of PV (Lo Muzio, et al., 2002, Mignogna, et al., 2001, Sharma, et al., 2007, Williamson, et al., 2006). Pg is closely related to β -catenin, an established regulator of the canonical Wnt/wingless signalling pathway (Huelsken and Birchmeier, 2001, Zhurinsky, et al., 2000). Plakoglobin is capable of associating and possibly competing with the same binding partners as β -catenin. Hence, through this competition Dsg3 is capable of modulating the Wnt signalling pathway through Pg (Gat, et al., 1998, Teulière, et al., 2004). Although studies have yielded some conflicting observations they do suggest that binding of auto-antibodies to Dsg3 induces intracellular signalling, including the altered localisation of Pg from desmosomes to modulate the transcription of TCF/LEF target genes, as well as the AP-1 target genes, including uPAR, c-Myc that may have implications in PV acantholysis (Caldelari, et al., 2001, Garcia-Gras, et al., 2006, Mignogna, et al., 2001, Seishima, et al., 1997, Williamson, et al., 2007, Williamson, et al., 2006). Findings from both PV and cancer specific studies are in agreement with the hypothesis that Dsg3 acts as an upstream regulator of AP-1 since the TCF/LEF transcription factors have also been reported to bind to the AP-1 promoter in a JNK-dependent manner (Nateri, et al., 2005) or directly interact with and activate cJun in cancer cells (Rivat, et al., 2003).

With respect to the Dsg3-dependent activation of cJun:AP-1, I provide evidence that, in addition to JNK and p38 MAPKs, PKC, ROCK, Src and Rac1 are possibly involved since inhibition of these signalling molecules resulted in abrogated Dsg3-dependent AP-1 activity as well as Ezrin activation. The involvement of PKC, ROCK, Src and Rac1 in the activation of AP-1 has been previously demonstrated in T lymphocytes (Baier-Bitterlich, et al., 1996, Funakoshi-Tago, et al., 2003, Kaminuma, et al., 2001). However, further study is necessary in order to elucidate the involvement of these molecules in Dsg3-dependent processes.

Ezrin has been identified as a transcriptional target of cFos:AP-1 activation (Jooss and Müller, 1995). I did not report an increase in Ezrin expression suggesting that Dsg3 may regulate cJun homodimers or heterodimers with other bZIP proteins such as ATF family transcription factors.

The role of cJun in the progression of cancer through the AP-1 invasion programme is somewhat conflicting since cJun has been shown to preferentially regulate genes

associated with proliferation as well as apoptosis while cFos is shown to preferentially modulate genes associated with angiogenesis and invasion of tumour cells (Eferl and Wagner, 2003). However, some of cJun target genes have established roles in the tumour invasion including MMP1/2/3/9 (Whitmarsh and Davis, 2000), Ezrin and CD44 (Katabami, et al., 2005) which confer invasiveness; uPAR (van Dam and Castellazzi, 2001) and VEGF (Schreiber, et al., 1999) involved in angiogenesis. Studies by Smith *et al.*, show that the overexpression of cJun promotes cell spreading, loss of cell polarity and increased motility and invasiveness with increased expression of MMP-9 in the breast cancer cell line, MCF-7 (Bos, et al., 1999, Smith, et al., 1999). Bos *et al.* also observed increased cell spreading, motility and invasion in chick embryo fibroblasts overexpressing cJun (Bos, et al., 1999), with the disruption of cJun reported to inhibit cell migration and invasiveness in mouse embryo fibroblasts (Jiao, et al., 2008) as well as in oral squamous cell carcinoma cell lines (Lu, et al., 2012). Together, these findings are consistent with the phenotype of A431 cells with Dsg3 overexpression implicating Dsg3-dependent activation of cJun:AP-1 in the regulation of cell migration and invasion. What remains undefined in the studies by Smith *et al.* and Bos *et al.* is the composition of the cJun:AP-1 dimer, which raises the possibility that migration and invasion could be elicited by cJun heterodimers with Fos, Maf and ATF proteins rather than cJun homodimers. It has been shown that cJun:cFos are more stable and have a higher affinity for the AP-1 response element, TRE, than cJun homodimers (Allegretto, et al., 1990). cJun can also form heterodimers with CREB/ATF proteins to bind the CRE response element. The dimeric partner of cJun have a significant effect on the cJun phenotype. For example, cJun:Fos confers anchorage-dependent cell growth while cJun:ATF confers growth-factor dependent cell growth with only a combination of these heterodimers resulting in a fully transformed phenotype (van Dam, et al., 1998). Taking into consideration the number of variables that attribute to the regulation of AP-1 activity, it would be almost impossible to predict the cJun:AP-1 composition and subsequent target gene repertoire in Dsg3 overexpressing cell lines. As a result further study aimed at defining cJun:AP-1 composition and its target gene repertoire in A431-D3 cell could enable conclusions to be drawn. However, since the cJun:cFos heterodimers have been shown to be required for cell invasion both *in vivo* (Saez, et al., 1995, Young, et al., 1999) and *in vitro* (Hennigan, et al., 1994, Lamb, et al., 1997, Malliri,

et al., 1998), I would speculate that the cooperative function by c-Jun and c-Fos likely plays a role in accelerated migration and invasion of the Dsg3 overexpressing cells.

Desmoglein 3 regulates cell morphology, migration and invasion through AP-1 and PKC-dependent Ezrin activation

The likelihood of a positive role of Dsg3 in cancer has been documented in several studies based on gene screening for potential biomarkers in cancers, identifying the upregulation of Dsg3, the pemphigus vulgaris antigen (PVA) in a variety of cancers including those of the skin, lung, prostate and head and neck (Fukuoka, et al., 2007, Hiraki, et al., 1996, Kurzen, et al., 2003, Patel, et al., 2013, Trojan, et al., 2005, Trojan, et al., 2005). Furthermore, the overexpression of Dsg3 has also been shown to correlate with the clinical stage of cancers suggesting its potential use as a therapeutic target in treating cancer and preventing tumour progression and metastasis (Chen, et al., 2007). Collectively, the phenotype of Dsg3 overexpressing cells, includes hyper-proliferation (Merritt, et al., 2002), increased membrane protrusions (Tsang, et al., 2010), loss of cell polarity (Tsang, et al., 2012), abnormal differentiation (Merritt, et al., 2002), increased motility and invasion (Chen, et al., 2013, Tsang, et al., 2012) are all consistent with observations in this study. The correlation between these phenotypes and level of Dsg3 overexpression together with its identification as a potential biomarker in the detection of lymph node metastasis in head and neck squamous cell carcinoma (Patel, et al., 2013) affirms its potential as a metastatic determinant and a valid target for anti-metastatic therapy (Chen, et al., 2007). This proposal is supported by two recently released patent publications in which Dsg3 has been used as a biomarker in cancer diagnosis and also a therapeutic target. However, the translational research regarding the role of Dsg3 as a potential target is lacking and warrants further in-depth investigations

To date, the only mechanistic study regarding the role of Dsg3 in cancer was conducted by Chen *et al.*, as mentioned above, this study based on RNAi knockdown of Dsg3 provides *in vitro* and *in vivo* evidence of the positive role of Dsg3 in cell invasion and the genetic metastatic programme in head and neck cancer. My reports are consistent with these findings with respect to both Dsg3 phenotype and mechanistic implications. With respect to Dsg3-dependent mechanisms, cross-talk between TCF/LEF and AP-1 pathways has been previously reported (Rivat, et al., 2003,

Saadeddin, et al., 2009) with both JNK and p38 capable of activating TCF transcription factors (Eferl and Wagner, 2003). Additionally the promoter of MMP-7, as highlighted in the study by Chen *et al.*, has a TATA box and binding site for both TCFs and AP-1 (Le Floch, et al., 2005, Pacheco, et al., 2002, Rivat, et al., 2003). This also implicates Pg in the Dsg3-dependent signalling axis, by demonstrating that the levels of Dsg3 expression directly affect Pg cellular localisation and subsequent interactions with TCF in the nucleus to suppress TCF/LEF transcriptional activity (Chen, et al., 2013). This study is, in part, consistent with a study by Tsang *et al* which demonstrated that Dsg3 co-Immunoprecipitates with Pg in a Dsg3-dose-dependent manner (Tsang, et al., 2010) to possibly sequester Pg to the plasma membrane, preventing its translocation to the nucleus, where it would interact and suppress TCF/LEF transcriptional activity. Nevertheless, this hypothesis warrants further investigation in A431-D3 cell before drawing a conclusion.

Interestingly, the basal localisation of Ezrin in epithelial cells has been reported to be aberrant as Ezrin adopts an apical localisation in polarised epithelial cells. Therefore, the basolateral localisation observed in this study suggests a loss of cell polarity which is consistent with the Dsg3 phenotype. Loss of cell polarity, coupled with the enhanced cell spreading, and enhanced migratory and invasive characteristics, is indicative of epithelial to mesenchymal transition (EMT), a process essential to cancer progression and metastasis (Tsai, et al., 2012), supporting further the proposal that Dsg3 is acting as a metastatic determinant .

My data suggest that Dsg3 regulates cell morphology, migration and invasion through AP-1-dependent transcriptional regulation of genes of the migration and invasion programme. For the successful translation of genetic programmes into a cellular phenotype, signalling pathways need to be modified to implement these processes. My results indicate that Dsg3 may facilitate this translation through the modulation of AP-1 target gene products. For example, I show that Dsg3 may recruit Ezrin to the basolateral membrane in order to co-ordinate the formation of locomotory and invasive membrane structures. I also show that silencing of Dsg3 inhibits the colocalisation of Ezrin with CD44 and F-actin at the plasma membrane. The Ly24, lymphocyte homing receptor, CD44, is another gene product of cJun:AP-1 and is shown to be required for AP-1-dependent invasion (Lamb, et al., 1997). Upon activation, Ezrin

acts as a molecular linker between CD44 and the actin cytoskeleton (Hirao, et al., 1996, Tsukita, et al., 1994, Yonemura, et al., 1998). CD44 is a transmembrane cell adhesion molecule which functions in process including cell homing, adhesion, angiogenesis, cell proliferation and migration (Martin, et al., 2003). Interestingly, variant isoforms of CD44 have been shown to be expressed exclusively in malignant cells and tumour-derived cell lines and CD44 expression has been shown to correlate with metastatic behaviour and poor prognosis in cancers including head and neck (Gotoda, et al., 2000), pancreatic (Gansauge, et al., 1995, Gotoda, et al., 1998) and lung cancers (Miyoshi, et al., 1997, Tran, et al., 1997). Furthermore, the interactions of CD44 and Ezrin has been proven to promote cancer cell migration and invasion (Donatello, et al., 2012).

I have identified several signalling molecules that are likely to be involved in the Dsg3-dependent activation of Ezrin and cJun:AP-1 which, when inhibited, abrogated Dsg3-dependent Ezrin activation and AP-1 transcriptional activity. In normal physiological conditions, the serine/threonine kinase PKC acts as an effective regulator in desmosome assembly with evidence that PKC activation by TPA induces the formation of desmosomes even in the presence of low calcium (Kitajima, et al., 1999). PKC has also been identified as downstream effector of Dsg3-dependent signalling with its activation and translocation to cortical actin reported in response to binding of Dsg3 autoantibodies in cultured keratinocytes (Osada, et al., 1997). In accord, Sanchez-carpintero *et al* demonstrated that the inhibition of PKC, PLC and calmodulin inhibit Dsg3-autoantibody-induced acantholysis *in vivo* (Sánchez-Carpintero, et al., 2004). In cancer, both in *vitro* and in *vivo* models show that PKC α and β isoforms exhibit increased activity associated with increased cell motility and invasion with their inhibition suppressing this phenotype (Engers, et al., 2000, Koivunen, et al., 2004, Masur, et al., 2001, Zhang, et al., 2004). Further, studies also suggest that PKC α -dependent invasion may occur through inhibition of adheren and desmosomal junctions (Koivunen, et al., 2004, Masur, et al., 2001). PKC is associated with and activates Ezrin (Ng, et al., 2001, Wald, et al., 2008), with the phosphorylation of Ezrin induced by PKC α resulting in enhanced cell migration and invasion (Ng, et al., 2001). In support of this observation Wald *et al* demonstrated that the atypical PKC isoform, iota, is essential to the activation and normal distribution of Ezrin at the early stages of intestinal epithelial cell differentiation (Wald, et al., 2008) and the colocalisation of

Ezrin with CD44 (Stapleton, et al., 2002). Furthermore, PKC is known to regulate AP-1 activity through its ability to activate its upstream regulators JNK and p38 MAPKs (Efimova and Eckert, 2000, Efimova, et al., 1998, Ghaffari-Tabrizi, et al., 1999, Reifel-Miller, et al., 1996, Sokolova, et al., 2013). These observations together with my results, support the potential role of PKC in Dsg3-dependent regulation of Ezrin and cJun:AP-1 activation.

Src, a non-receptor tyrosine kinase, is a member of the Src family kinases. Src is activated following stimulation of membrane receptors and integral to the maintenance of normal cell homeostasis through the regulation of functions including proliferation, adhesion, cell shape change, actin cytoskeleton and migration (Thomas and Brugge, 1997, Yeatman, 2004). Overexpression or hyper-activation of Src is associated with a variety of carcinomas, including colorectal, breast, head and neck and lung (Shor, et al., 2007, Summy and Gallick, 2003). While its transforming ability is unlikely (Biscardi, et al., 1999) its role in tumour progression and metastasis is well established, with increased Src activity associated with hyperproliferation, migration, invasion and metastasis (Guarino, 2010, Irby and Yeatman, 2000). Recent studies by Tsang *et al.* showed that overexpression of Dsg3 in A431 cells causes a significant increase in Src activation contributing to filopodia formation and cell migration (Tsang, et al., 2012, Tsang, et al., 2010). I report that Src activation also contributes to Dsg3-dependent cJun:AP-1 activation since its inhibition suppressed Dsg3-dependent cJun:AP-1 transcription most likely occurs through the regulation of AP-1 MAPK pathway as Src has been identified as upstream of p38, JNK and ERK to promote the transcription of target genes of the migration and invasion programme (Guarino, 2010). I also demonstrate that the overexpression of Dsg3 increases the phosphorylation of various Src family kinases, compared to Vect Ct (Figure 7.1). Src functions at the plasma membrane to facilitate cell migration, invasion and actin reorganisation (Guarino, 2010) and could play a key role in aiding Dsg3-dependent migration and invasion not only on a transcriptional level via Src signalling but also through the regulation of locomotory and invasive machinery at the leading edge through the association of Src with integrin, paxillin and Rho GTPases (Guarino, 2010).

RhoA has been shown to play a positive role in the regulation of desmosomal adhesion with the treatment of keratinocyte cultures with Dsg3-autoantibodies resulting in its

inactivation in a p38-dependent manner (Waschke, et al., 2006). RhoA is capable of activating the ERM family proteins (Yonemura, et al., 2002) as well as co-localising with cortical actin and Ezrin at membrane ruffles and lamellipodia of spreading and migrating endothelial cells (Menager, et al., 1999). RhoA can also function downstream of Ezrin, in a positive feedback loop, to regulate the organisation of the actin cytoskeleton (Hatzoglou, et al., 2007). Studies by Spindler *et al.* suggest the involvement of Rho GTPases as part of the pathological mechanism of PV, with reductions in their activity causing loss of cell adhesion, with RhoA having the most protective effect in cell adhesion (Spindler and Waschke, 2011). In line with this finding, I have shown that inhibition of RhoA results in significant inhibition of Dsg3-dependent Ezrin activation, suggesting that RhoA participates in the Dsg3/Ezrin signalling axis. Like RhoA, Rac1 is another small GTPase that has been implicated in numerous cellular processes including cell-cell adhesion, actin based motility and epithelial differentiation. Inhibition of Rac1 also showed a significant suppression of the Dsg3-dependent cJun-AP-1 transcriptional activity and Ezrin phosphorylation. In support, Dsg3 has recently been reported to regulate Rac1/Cdc42 activity to promote actin organisation and dynamics (Tsang, et al., 2012).

Taken together my study is consistent with the notion that Dsg3 acts as a potent regulator of various signalling pathways that function in concert to modulate actin based cellular events in cancer.

8.2 Conclusion

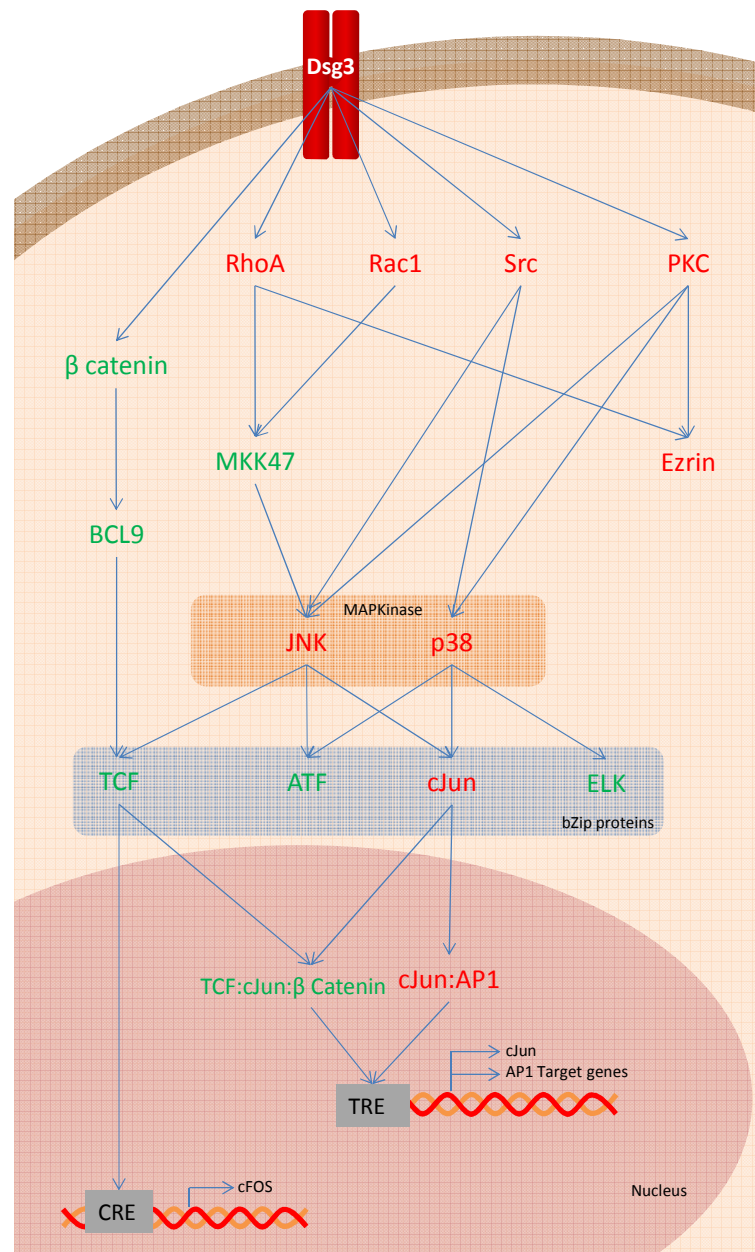


Figure 8.2 Proposed mechanism of Dsg3-dependent regulation of PKC-dependent activation of Ezrin and cJun:AP-1. Pathways highlighted in red are supported by the findings presented in this thesis. Pathways highlighted in Green are established mechanisms published in the literature. As shown, the downstream effectors of Dsg3 include RhoA, Rac1, PKC and Src that facilitate the activation of Ezrin and cJun:AP-1 activity.

Figure 8.2 summarises the major findings described in this thesis with regard to Dsg3 in the regulation of intracellular signalling (red) and in relation to the established pathways (green), highlighting the proposed function of Dsg3 as a cell surface regulator of cell morphology, migration and invasion through the regulation of cJun:AP-1 and PKC-dependent Ezrin activation. This proposal is consistent with and supported by many studies reported in literature that demonstrate the signalling

capabilities of Dsg3 and those that have identified Dsg3 to be unregulated in cancer. This study however, goes further to elucidate the molecular mechanisms by which Dsg3 promotes cancer cell migration and invasion. I have shown that Dsg3 possibly achieves this through regulating the transcription of genes of the migration and invasion programme through cJun:AP-1. Furthermore, I show that Dsg3 may fulfil an orchestrative function in promoting cell migration and invasion through a complex with a cohort of membrane associated proteins including Ezrin that in turn facilitates the formation of locomotory and invasive structures. In conclusion, this study intrinsically links a recently patented biomarker of lymph node metastasis, Dsg3, to cJun:AP-1 and Ezrin, both of which have been individually implicated in cancer progression and metastasis.

8.3 Future perspective

The identification of increased Dsg3 expression and increased expression and/or activation of Ezrin in cancer progression and metastasis are important events which assist in distinguishing the differences between normal, non-metastatic and metastatic malignant cells. However, to translate these observations into tangible anti-metastatic therapeutic targets requires a greater understanding into how these aberrations in protein expression and activation alter cell network biology, how and why they are overexpressed and hyperphosphorylated. Such an understanding of metastatic determinates, or molecules associated with metastasis will aid our understanding of refractory metastases and acquired drug resistance mechanisms which currently threatens the success and longevity of current therapies such as those targeting HER2 in breast cancer, BRAF in melanoma and ALK in lung cancers which is evident in drug resistance that often occurs in metastatic relapse. The treatment of metastatic disease is thought to be complicated by differential expression and/or activity of oncogenes and/or the influence of the secondary site microenvironment (Wan, et al., 2013).

Though a novel gain-of-function approach detailed examination of the effect of Dsg3 overexpression on the cellular proteome and phenotype has demonstrated Dsg3s ability to regulate gene transcription through AP-1 and participate in the organisation of specialised regulatory protein complexes involving the actin regulator, Ezrin. Through this novel perspective, our understanding, as to how the Dsg3 is able to promote cancer progression and metastasis, is expanded and supports the proposal of Dsg3 as a potential therapeutic target (Chen, et al., 2007).

To further develop the presented finding towards translational application. The validation of the major findings *i.e.* the phenotype, hyper-phosphorylation of Ezrin and enhanced AP-1 activity associated with Dsg3 overexpression, will be essential. This could be achieved through the analysis of Primary cancer cells which exhibit increased Dsg3 expression. it would be interesting and necessary to expand this study in primary cells derived from SCC as well as those from the metastatic sites and to compare their gene expression and proteomic profile with respect to Dsg3. A recent study based on oesophageal SCC has shown that in addition to strong correlation between Dsg3 expression and histological grade, the cytoplasmic distribution of Dsg3 in the absence of lymph node metastases predicts worse overall survival than those with membrane-

cytoplasmic staining (Fang, et al., 2014). Thus, an increased protein solubility of Dsg3 might be a predictive factor of malignancy and prognosis that merits further confirmation. Importantly, the clinical implication of the work presented here will be dependent on replication of major findings *in vivo*.

Dsg3 is a transmembrane protein which associates with other desmosomal cadherins to confer cell-cell adhesion. The non-junctional pool of Dsg3 is membrane associated but not incorporated into desmosomes (Jennings, et al., 2011). It would be interesting to see if in malignant tissue overexpressing Dsg3 whether the increase is that of the Triton soluble or insoluble pool. Based on this study and that of other carried out by Dr Wan *et al.*, I would expect to see an increase in the soluble non-junctional pool of Dsg3.

Another aspect that would be interesting to examine would be the effect the microenvironment has on Dsg3 dependent signalling mechanisms, specifically if Dsg3 signalling and associated phenotype is influenced by changes in the microenvironment which may suggest that Dsg3 is functioning as a mechano-sensor. A preliminary study on this proposal in SCC is currently underway in this laboratory

Finally the development of this project to incorporate *in vitro* and *in vivo* live cell imaging to examine the localisation of Dsg3, Ezrin and other proteins of interest with respect to cellular events in real time and space would be invaluable. Such a model, enlisting techniques such as intra-vital imaging with FRET and FLIM would allow us to look at recruitment, interactions and phosphorylation status in great detail in addition to the localisation and sequent of these events. Interactions with microenvironment could also be examined, particularly its effect on cellular behaviour.

In conclusion, this thesis provides important insights into the possible mechanisms by which Dsg3 promotes cancer cell migration, invasion and metastasis affirming Dsg3's role in cancer cell biology and warrants further investigation into Dsg3 as a potential therapeutic target.

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